Influence of T Regulatory Cells on Humoral Immunity and Induction of Peripheral Tolerance

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2 Abbreviations

Ab	antibody
Ag	antigen
ADCC	antibody-dependent cell-mediated cytotoxicity
APC	antigen-presenting cell
BCR	B cell receptor
CD	cluster of differentiation
CSR	class switch recombination
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
DC	dendritic cell
DNA	deoxyribose nucleic acid
DTH	delayed type hypersensitivity
EDTA	ethylenediaminetetraacetic acid
GITR	glucocorticoid-induced tumor necrosis factor receptor
HDM	house dust mite
HR1	histamine receptor 1
HR2	histamine receptor 2
HR3	histamine receptor 3
HR4	histamine receptor 4
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
IPEX	immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome
LC	Langerhans' cell
LPS	lipopolysacharide
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NFκB	nuclear factor κB
NK	natural killer
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PD-1	programmed death-1
SHM	somatic hypermutation

SIT	specific immunotherapy
TCR	T cell receptor
TGF-β	transforming growth factor-β
TLR	Toll-like receptor
Th	T helper cell
Tr1	type 1 regulatory T cell
Treg	T regulatory

3 Summary

Activation of the immune system and production of immunoglobulin (Ig) E against innocuous environmental antigens leads to allergic diseases. The non-inflammatory antibody isotypes IgG4 and IgA play an important role in neutralisation and subsequent clearance of antigens. It is still not clearly known why some people develop allergic reactions and others do not, but the balance between allergen-specific T helper (Th) 2 cells and the IL-10-secreting T regulatory type 1 (Tr1) cells seems to be very important. Whereas healthy individuals have a higher frequency of allergen-specific Tr1 cells, allergen-specific Th2 cells are more prevalent in allergic people. Tr1 cells show a suppressive effect on T cell activation, are induced in the periphery during specific immunotherapy (SIT) and therefore are very important for induction and maintenance of peripheral tolerance. Another subset of regulatory T (Treg) cells comprises $CD4^+CD25^+FoxP3^+$ T cells, which have similar features as Tr1 cells, they are called natural Treg cells since they originate in the thymus, however, they can also be generated in the periphery.

Since it is well known that B cells need Th1- or Th2 cell-help to generate Igs, we investigated whether regulatory T cells also influence Ig production. We showed that both IL-10-secreting Tr1 and $CD4^+CD25^+$ Treg cells suppress IgE, but induce IgG4 production, however, production of IgA was not affected. Very interestingly, IgA induction was stongly dependent on activation of the innate immune system, specially TLR7 or TLR9 stimulation on B cells, and did not require T cell help. This could endow the human system with a very fast immune response to combat invading pathogens.

For better understanding the mechanisms of T cell tolerance, we investigated the immune response of several non-allergic beekeepers, which received high doses of bee venom via natural bee stings during their occupation. We could observe that continuous exposure to high doses of bee venom antigens leads to a clonal switch of venom antigen-specific Th1 and Th2 cells towards IL-10-secreting Tr1 cells. These high numbers of antigen-specific Tr1 cells persisted as long as antigen was present, however, they returned to initial levels two to three months after the last bee sting. Furthermore, histamine receptor 2 (HR2), which is predominantly expressed on effector Th2 cells, is upregulated and directly suppresses activated Th2 cells and induces IL-10 production by DCs, Treg and Th2 cells.

We conclude that Tr1 cells, which are increased during SIT, as well as $CD4^+CD25^+$ Treg cells contribute to the control of allergen-specific immune

responses in several ways: I) Suppression of antigen-presenting cells that support the generation of effector Th2 and Th1 cells; II) suppression of Th2 and Th1 cells; III) suppression of mast cells, basophils and eosinophils; IV) interaction with resident tissue cells and remodeling, and, as shown in this study, V) induction of the non-inflammatory antibody isotype IgG4 and suppession of the inflammatory antibody isotype IgE. In addition, we could gain more insight into the mechanisms of peripheral tolerance, which is crucial for a healthy immune response.

Induction of Treg cells or the increase of their suppressive capacity by drugs, cytokines or costimulatory molecules is an important target not only for the treatment of allergy and asthma, but also for transplantation and autoimmunity.

4 Zusammenfassung

Die Aktivierung des Immunsystems und die damit verbundene Bildung von Immunglobulin (Ig) E gegen normalerweise harmlose Umweltantigene führt zu allergischen Erkrankungen. Die Immunglobuline IgG4 und IgA mit ihren anti-inflammatorischen Eigenschaften spielen eine wichtige Rolle bei der Neutralisierung und Beseitigung eines Antigens. Der Grund, warum ein Antigen in einigen Personen eine allergische Reaktion auslöst und in anderen nicht, ist noch nicht genau bekannt. Man weiss jedoch, dass das Gleichgewicht zwischen allergen-spezifischen T-Helfer 2-Zellen (Th2) und den IL-10-sekretierenden regulatorischen T-Zellen (Tr1) entscheidend ist. Gesunde Personen haben mehr allergen-spezifische Tr1-Zellen, Allergiker hingegen besitzen mehr allergen-spezifische Th2-Zellen. Tr1-Zellen unterdrücken aktivierte Th-Zellen und werden während der allergen-spezifischen Immuntherapie (SIT) gebildet. Das zeigt, dass sie sehr wichtig für die Induktion und Erhaltung der peripheren Toleranz sind. Die $CD4^+CD25^+FoxP3^+$ T-Zellen gehören ebenfalls zu den regulatorischen T (Treg) Zellen, bilden jedoch eine eigenständige Population. Sie besitzen ähnliche Eigenschaften wie Tr1-Zellen, werden jedoch natürliche Tregs genannt weil sie im Thymus gebildet werden, aber sie können auch in der Peripherie entstehen.

Da seit langem bekannt ist, dass B Zellen zunächst durch T-Helfer 1 (Th1)- oder Th2-Zellen aktiviert werden müssen um Antikörper zu produzieren, untersuchten wir, ob auch Tregs einen Einfluss auf die Antikörperbildung haben. Wir zeigten, dass sowohl die Tr1-Zellen als auch die $CD4^+CD25^+$ Treg-Zellen die IgE Produktion unterdrückten, die IgG4 Bildung jedoch erhöhten, die IgA Produktion hingegen war nicht beeinflusst. Interessanterweise wurde IgA durch die Aktivierung des angeborenen Immunsystems induziert. Dies geschah durch die Stimulierung der Toll-like Rezeptoren (TLR) 7 und 9 auf den B Zellen und ohne Hilfe der T-Zellen. Dieser Abwehrmechanismus könnte dem menschlichen Körper dazu dienen, eindringende Krankheitserreger schnell zu beseitigen.

Um die Mechanismen der T-Zelltoleranz besser zu verstehen, untersuchten wir die Immunantwort bei nicht-allergischen Imkern, die durch Bienenstiche hohe Dosen Bienengift erhalten hatten. Anhaltende hohe Bienengiftkonzentrationen hatten einen klonalen Wechsel von antigen-spezifischen Th1- und Th2-Zellen hin zu IL-10-sekretierenden Tr1-Zellen zur Folge. Die erhöhte Anzahl antigen-spezifischer Tr1-Zellen blieb bestehen, solange das Antigen präsent war. Zwei bis drei Monate nach dem letzten Bienenstich ging die Anzahl der Tr1-Zellen jedoch wieder auf

anfängliche Werte zurück. Ausserdem wurde der Histaminrezeptor (HR) 2, der vor allem auf den Th2-Zellen exprimiert wird, hochreguliert. Dies führte zur Hemmung aktivierter Th2-Zellen und zur IL-10 Produktion durch dendritische Zellen, Tregs und Th2-Zellen.

Wir kommen zum Schluss, dass sowohl Tr1-Zellen, welche während der SIT gebildet werden, als auch die $CD4^+CD25^+$ Treg-Zellen eine wichtige Rolle bei der Kontrolle der allergen-spezifischen Immunantwort spielen und zwar indem sie: I) Antigen-präsentierende Zellen, welche für die Generierung der Th1- und Th2-Zellen verantwortlich sind, unterdrücken; II) Th2- und Th1-Zellen unterdrücken; III) Mastzellen, Basophile und Eosinophile unterdrücken; IV) mit residenten Gewebszellen und dem Remodelling interagieren und, wie wir hier zeigen konnten, V) die nicht-inflammatorischen IgG4 Antikörper induzieren, die inflammatorischen IgE Antikörper jedoch supprimieren. Zudem konnten wir einen tieferen Einblick in die Mechanismen der peripheren Toleranz gewinnen, die für eine normale Immunantwort äusserst wichtig ist.

Induktion der Treg-Zellen oder die Verstärkung ihrer suppressiven Kapazität durch Medikamente, Zytokine oder co-stimulatorische Moleküle wäre nicht nur für die Behandlung von Allergien und Asthma von Vorteil, sondern könnte auch bei Transplantationen und Autoimmunkrankheiten eingesetzt werden.

5 Introduction

5.1 History

In 1906, the Austrian paediatrician Clemens von Pirquet observed in his patients an “altered reactivity” when they were exposed to different environmental substances and therefore, he created the term “allergy” (Greek *allos*, other; *ergon*, energy/response). Later, these environmental substances were called “allergens”. Allergy is a tissue-damaging immune response and involves both a foreign substance (allergen) and a genetic susceptibility. Affected individuals can produce a specific class of antibody, namely IgE, which is responsible for the majority of manifestations seen in patients with allergic diseases.

In 1890, Emil von Behring and Shibasaburo Kitasato discovered antibody activity against diphtheria and tetanus toxins. Behring and Kitasato proposed that a mediator in serum could react with a foreign antigen (1). Seven years later, Behring and Kitasato described a "lock-and-key" interaction between receptors and specific toxins on the surface of cells and hypothesized this binding as the trigger for the antibody production (2). Almroth Wright suggested in 1904, that soluble antibodies could label bacteria by coating them, and therefore render them susceptible for phagocytosis and killing; e termed this process opsonization (3).

In the 1920s, Michael Heidelberger and Oswald Avery showed that antigens could be precipitated by antibodies. They also discovered that antibodies were made of protein (4). The lock-and key theory proposed by Ehrlich was confirmed by Linus Pauling in the 1940s (5). In 1948, Astrid Fagreaus observed that plasma cells could produce antibodies (6) and Gerald Edelman and Joseph Gally discovered the antibody light chain in the early 1960s (7). Thereafter, Edelman could show that antibodies are composed of disulphide bond-linked heavy and light chains. Around the same time, antibody-binding (Fab) and antibody tail (Fc) regions of IgG were characterized by Rodney Porter (8). Together, these scientists suggested the structure and complete amino acid sequence of IgG, and were awarded the Nobel prize in Physiology and Medicine in 1972 (8). Also other immunoglobulin isotypes were discovered in the 1960s: Thomas Tomasi found secretory antibody (IgA) (9), Rowe and Fahey identified IgD (10), and IgE was discovered by Kikishige and Teruki Ishizaka (11).

In 1963, Coombs and Gell established a classification of hypersensitivity reactions upon differences in the mechanisms, cells, and mediator molecules involved. Three types of hypersensitivity belong to the humoral branch and are mediated by antibody or antigen-antibody complexes. A forth type is part of the cell-mediated branch (Table 1).

Table 1. Hypersensitivity responses

	Type I	Type II	Type III	Type IV
Immune mechanisms	IgE-mediated (Immediate hypersensitivity)	IgM-, IgG-mediated	Immune complex-mediated (IgM, IgG)	T cell-mediated
Antigen	Soluble antigen	Cell- or extracellular matrix-associated antigen	Soluble antigen	Soluble or cell-associated antigen
Effector mechanisms	Crosslinking of IgE bound on mast cells and basophils and subsequent degranulation	Opsonization and phagocytosis of cells, complement activation or antibody-dependent cell-mediated cytotoxicity (ADCC)	Ag-Ab complexes deposited in various tissues induce complement activation and infiltration of neutrophils	Sensitized Th1 cells release cytokines that activate macrophages or CTLs which mediate cell killing
Clinical manifestations	Systemic anaphylaxis, allergic rhinitis, asthma, food allergies, eczema, hives	Blood transfusion reactions, some drug allergies (e.g. penicillin), tissue injury	Arthus reaction, serum sickness, systhemic lupus erythematosus, vasculitis	Contact dermatitis, graft rejection
Time	Within minutes	Takes hours to a day	Takes hours, days or weeks	Takes two to three days to develop

Adapted from Abbas and Lichtman, Cellular and Molecular Immunology. Fifth ed. Philadelphia: Saunders 2003, Goldsby et al., Immunology. Fifth ed. New York: Freeman 2003.

5.2 The adaptive immune system

The immune system is divided into the innate immunity, which is less specific and into the adaptive immunity, which is very specific. The first line of defense against infection is provided by the innate immunity. The adaptive immunity develops as a response and adapts to the infection, and is characterized by outstanding specificity for distinct molecules and an ability to remember and respond more efficiently to repeated exposures to the same microbe. Furthermore, it has an extraordinary capacity to distinguish among very closely related microbes and molecules. There are two types of adaptive immune responses, called humoral immunity and cell-

mediated immunity. Humoral immunity is performed by antibodies that are produced by B cells. Cell-mediated immunity, which is performed by T cells, is responsible for the destruction of microbes that reside in phagocytes or for the killing of infected cells.

5.2.1 T helper cells

The generation of allergen-specific $CD4^+$ T helper cells (Th) is the initial event which is responsible for the development of allergic diseases. In response to different types of microbes, naive $CD4^+$ T cells differentiate into distinct subsets of effector cells that produce different cytokines and therefore can perform diverse effector functions. Currently, T helper 1 (Th1) and T helper 2 (Th2) cells are the best defined subsets of effector T cells of the $CD4^+$ helper lineage. The present understanding is that interleukin-4 (IL-4), which activates the transcription factors GATA3 and STAT6, induces the differentiation of naive T cells activated by antigen-presenting cells (APCs) into Th2 cells (12-14). These effector Th2 cells produce various cytokines like IL-4, IL-5 and IL-13, which are responsible for several effector functions: production of allergen-specific IgE by B cells, development and recruitment of eosinophils, production of mucus and contraction of smooth muscles as well as tissue homing of Th2 cells (12,13,15,16). Furthermore, IgE-mediated cross-linking of receptors and subsequent degranulation of basophils and mast cells is the main event in type I hypersensitivity, which may lead to allergic inflammation.

Many intracellular bacteria, viruses and some parasites elicit innate immune reactions. DCs are induced to produce IL-12. IL-12 binds to receptors on antigen-stimulated $CD4^+$ T cells and activates the transcription factor STAT4 and T-bet, which promote the differentiation of T cells into Th1 cells. The Th1 subset is characterized by the secretion of interferon-γ (IFN-γ), the principal macrophage-activating cytokine, and has important functions in innate and adaptive cell-mediated immunity. Importantly, Th1 cells may contribute to chronicity and effector phase in allergic diseases, although Th2 cells are responsible for the development of allergic diseases (17-22). For instance, IFN-γ up-regulates Fas on keratinocytes and makes them susceptible to apoptosis, which is an essential pathogenetic event in eczematous dermatitis (18,23). In asthma, tumour necrosis factor-α (TNF-α) and IFN-γ cause epithelial shedding because they induce bronchial epithelial apoptosis (18).

Not only the effector functions, but also development and expansion of the Th1 or Th2 subsets are dependent on their produced cytokines. IFN-γ secreted by Th1 cells enhances further Th1 differentiation and prevents the proliferation of Th2 cells. IL-4 produced by Th2 cells promotes Th2 differentiation and inhibits activation of Th1 cells. Therefore, each subset amplifies itself and simultaneously, counter-regulates the other subset (24).

5.2.2 B cells

B cells are antibody-producing cells, which play an important role in the humoral immunity. They mature within the bone marrow and when they leave, each B cell expresses a unique antigen-binding antibody molecule on the cell surface, which is also called B-cell receptor (BCR). An immature B cell, which has never been exposed to an antigen is known as naive B cell, and expresses only cell surface bound IgM. When reaching maturity, these cells start to express both IgM and IgD with identical antigen binding regions, and are ready to respond to antigen (25). When such a B cell first encounters an antigen that matches its membrane-bound receptor, the B cell starts to proliferate rapidly; its progeny differentiate into memory B cells and effector B cells termed plasma cells. Approximately 60% of naive B lymphocytes and 40% of memory B lymphocytes are found in human peripheral blood (26). Memory B cells have a longer lifespan than naive cells and survive for several years. This allows the immune system to remember an antigen and respond faster upon future exposures (27). In contrast, naive B cells live only for about 6 weeks in humans or 1-3 months in mice. Furthermore, memory B cells express the same BCR as their parent B cell. Plasma cells are specialized to secrete huge amounts of the antibodies produced and they express little or no membrane-bound antibody. It is believed that a single plasma cell can secrete more than 2000 molecules per second (28).

Membrane IgD expression has been used in several studies to distinguish between naive and memory B lymphocytes (29,30). Both subsets can be further separated according to the expression of IgA, IgD, IgG, and IgM. All naive B lymphocytes are $IgD^{+}IgM^{+}CD27^{-}$. Memory B lymphocytes, however, can be $IgD^{+}IgM^{+}CD27^{+}$, $IgD^{-}IgM^{+}CD27^{+}$, or $IgG^{+}/IgA^{+}CD27^{+}$, representing, respectively, 15, 10, and 15% of circulating B lymphocytes (26).

5.2.2.1 Antibodies

Antibodies are found in blood or other body fluids of vertebrates. They are Y-shaped glycoproteins and their main function comprises identifying and neutralizing foreign objects, such as bacteria and viruses. Each molecule consists of two large heavy chains and two small light chains. Two identical heavy chains and two identical light chains, which are arranged into two Fab regions and an Fc region and are separated by a flexible hinge region, compose the monomeric structural and basic functional unit of an immunoglobulin (Fig. 1). An antigen is recognized by the paired variable regions at the tips of the Fab arms, while the Fc region interacts with various receptors and effector molecules. Antibodies are also called immunoglobulins, because the heavy and light chains have a characteristic tertiary structure of antiparallel β sheets, which are termed globular domains. Based on differences in the heavy chain C regions, five distinct antibody isotypes have been identified in mammals, known as IgA, IgD, IgE, IgG and IgM, which perform different roles, and help to elicit the appropriate immune response against different types of foreign objects they encounter (31). Secreted antibodies can also be dimeric with two Ig units like IgA or pentameric with five Ig units, like mammalian IgM (32). Random combinations of gene segments that encode different antigen binding sites (or paratopes), followed by random mutations in this area of the antibody gene create a huge and diverse population of antibodies (33). Furthermore, antibody genes also reorganize in a process, which is called class switching. During this process, a variable region from one type of heavy chain is relocated to another, therefore creating another isotype of the antibody. By this means can a unique antigen-specific antibody be used by several different parts of the immune system. Therefore, antibodies are an essential component of the adaptive immune system that adapts and remembers responses to invading pathogens. Antibody production is the main function of the humoral immune system (34).

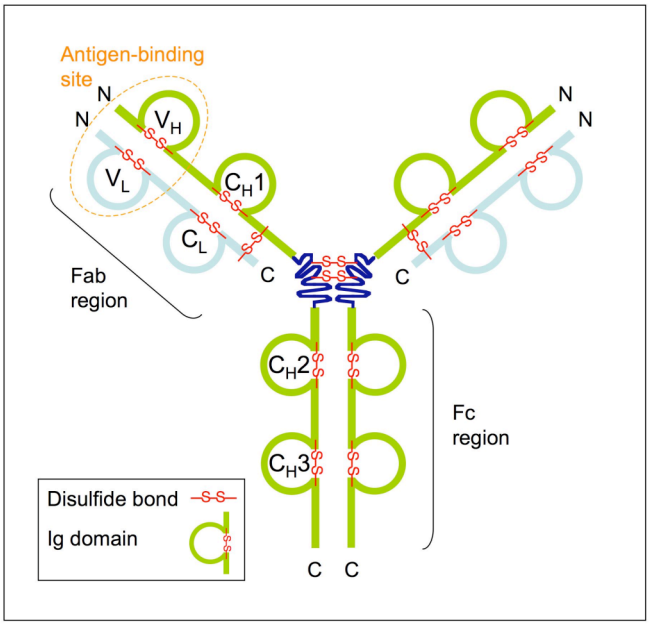

Figure 1. **Schematic diagram of an antibody molecule.** V_L: variable light chain; V_H: variable heavy chain; C_L: light chain constant region; C_H: heavy chain constant region.

5.2.2.2 Class switching

Class switching proceeds in the heavy chain gene locus by a mechanism called class switch recombination (CSR), which allows different daughter cells from the same activated B cell to produce different antibody isotypes. Initiation of this process requires two signals. The first one is delivered by cytokines, which target specific C_H genes for transcription, the second one is delivered in the case of T-dependent antigens by interaction of CD40 on B cells with its ligand CD40L on activated T cells (35). Triggering of CD40 on B cells by CD40L activates NF-κB-Rel (36), which binds to *cis*-acting κB elements within intronic I_H promoters 5' of switch (S) regions and leads to germline I_H-S-C_H transcription (37). S regions are conserved nucleotide motifs, which are found in DNA upstream of each constant region gene (except in the δ-chain). Germline transcription induces chromatin opening and facilitates the recruitment of activation-induced cytidine deaminase (AID). Deamination of cytosine residues in single-stranded DNA in the S regions by the AID leads to S region double-stranded breaks (38-40). Recombination of the two S regions and deletion of

the DNA-loop juxtaposes the somatically recombined V_HDJ_H exon to another C_H gene (41,42). Cells producing one Ig isotype have deleted all rearranged C_H genes 5' of this isotype. During class switching, only the constant region of the antibody heavy chain changes. The variable regions, however, and therefore antigen specificity, remain the same. Thus the progeny of a single B cell can produce antibodies, which are all specific for the same antigen, but these antibodies have the ability to change the effector function towards an appropriate antigenic challenge. The isotype generated depends on which cytokines are present in the B cell environment (43).

5.2.2.3 Antibody isotypes

5.2.2.3.1 Immunoglobulin G (IgG)

The most abundant isotype in serum is IgG, which constitutes about 80% of the total serum immunoglobulin. Four human IgG subclasses exist, and they are numbered according to their decreasing average serum concentrations: IgG1, IgG2, IgG3, and IgG4. A different size of the hinge region and the number and position of the interchain disulfide bonds between the heavy chains make these four molecules distinguishable between each other and affect the biological activity of the molecule.
IgG1 is able to cross the placenta and to protect the developing fetus. It has a high affinity to Fc receptors on phagocytic cells and therefore is very efficient in opsonizing foreign molecules. It has an intermediate capacity to activate complement.

IgG2 does not cross the placenta and because of the very low affinity to Fc receptors, it is weak in activating complement and opsonizing molecules.

IgG3 has the same features as IgG1, but it is the most efficient activator of the complement (28).

IgG4 has an intermediate affinity for Fc receptors. It is a monovalent, non-inflammatory antibody isotype because the half-molecules (one H plus one L-chain) can separate and pair again. Hence, bispecific antibodies are formed, which are unable to form large immune complexes and have a low potential to induce inflammation (44). Furthermore, IgG4 does not fix complement and as a blocking antibody, it competes with IgE antibodies for allergen and thus dampens IgE-mediated immune reactivity. Only when two antigens are present at the same time in the body and high levels of IgG4 antibodies are induced, significant amounts of bispecific antibody will be generated. The breaking of inter-heavy chain disulfide bonds, which are located in the hinge region, requires a reducing environment and is

believed to occur in the extracellular milieu, in blood or at cell surfaces. Furthermore, another important antibody heavy chain interface, which needs to be broken for the half-molecule exchange, is located between the C_H3 domains (45).

There are 5 different types of Fcγ receptors (FcγR) existing, which have different affinities for heavy chains of different IgG subclasses. So far, two general classes of FcγR have been identified: the activation receptors, FcγRI (CD64; $K_d \sim 10^{-9}$M), FcγRIIA (CD32; $K_d \sim 10^{-7}$M), FcγRIIIA (CD16; $K_d \sim 10^{-6}$M), FcγRIIIB (CD16; $K_d \sim 10^{-6}$M), characterized by the presence of a cytoplasmic ITAM sequence either intrinsic to the receptor or as part of an associated subunit, the γ or ζ chain, and the inhibitory receptor, FcγRIIB (CD32; $K_d \sim 10^{-6}$M) characterized by the presence of an ITIM sequence. Most effector cells of the immune system express activation FcγRs, notably monocytes, macrophages, NK cells, mast cells, eosinophils, neutrophils, and platelets, while they are not found on lymphoid cells. With the exception of T cells and NK cells, FcγRIIB is expressed on all cells of the immune system, and it is the only Fc receptor on B cells where it inhibits BCR-generated calcium mobilization and cellular proliferation (46,47). The activation receptors trigger different effector responses such as degranulation, phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), transcription of cytokine genes and release of inflammatory mediators (48-50). Normally, activation and inhibitory receptors are coexpressed on the cell surface and function in concert. Therefore, the outcome of the physiological response is the interplay of the contrary activation and inhibitory signaling pathways, and is determined by the level of expression of each receptor and the selective avidity of the IgG ligand. The expression and thus also the ratio of activating to inhibitory Fc receptors is regulated by exogenous factors. Cytokines like IL-4, IL-10, or TGF-β upregulate FcγRIIB, thereby, thresholds for cell activation are high, whereas inflammatory cytokines downregulate the inhibitory and upregulate the activating FcRs. However, that cytokine-mediated regulation of FcR expression is cell type-specific. Moreover, FcγRIIB has been suggested to play an important role in maintaining periperal tolerance. Homo-aggregation of FcγRIIB leads to B cell apoptosis via another signaling pathway and is responsible for deletion of low-affinity or self-reactive B cells that have undergone somatic hypermutation (SHM) (51).

5.2.2.3.2 Immunoglobulin A (IgA)

Although IgA constitutes only 10%-15% of the total immunoglobulin in serum, it is the predominant immunoglobulin class in external secretions such as breast milk, saliva,

tears, and mucus of the bronchial, genitourinary, and digestive tracts. These mucous membrane surfaces are the main entry sites for most pathogenic organisms. Secretory IgA, which is found in external secretions, consists of a dimer or tetramer, a J-chain polypeptide, and a polypeptide chain called secretory component. The secretory component comprises five immunoglobulin-like domains that bind to the Fc region domains of the IgA dimer. It is a fragment of the poly-Ig receptor, which transports polymeric IgA across cell membranes, and is expressed on the basolateral surface of most mucosal epithelia as well as on glandular epithelia in the mammary, salivary, and lacrimal glands. The secretory component masks sites susceptible to protease cleavage in the hinge region, allowing the IgA molecule to exist longer in the protease-rich mucosal environment. Pentameric IgM is also transported into mucous secretions by this mechanism. IgA-secreting plasma cells reside along mucous membrane surfaces. Every day, a human secretes from 5 g to 15 g of secretory IgA onto mucous secretions, where it can cross-link large antigens with multiple epitopes. The most important function of secretory IgA is binding to bacterial and viral surface antigens, so that the pathogens are not able to attach to the mucosal cells, thus avoiding viral infection and bacterial colonization. Complexes of secretory IgA and antigen are finally eliminated by the ciliated epithelial cells of the respiratory tract or by peristalsis of the gut (28).

In humans, two subclasses of IgA, IgA1 and IgA2, exist, which are encoded by separate genes. Several sequence differences are found in their heavy chain constant regions. A major difference between the subclasses has been discovered in the hinge region, which is very extended in IgA1. Serum IgA exists mainly as a monomer, predominantly (~90%) of the IgA1 subclass and is produced in the bone marrow, while polymeric secretory IgA consists mainly of the IgA2 subclass.

Human FcαRI (CD89) is constitutively expressed on neutrophils, monocytes, eosinophils, some macrophages, interstitial DCs, and Kupffer cells. In blood and tissues, neutrophils make up the majority of FcαRI-positive cells (52). Peritoneal fluid and bronchoalveolar lavage fluid were found to contain newly emigrated $CD89^+$ macrophages (52). Binding of antigen-complexed IgA to FcαRI elicits distinct biological responses depending on the cell type involved. These responses include antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, release of cytokines, superoxide generation, calcium mobilization, degranulation, and antigen presentation (53). In a recent study, it has been shown that crosslinking of FcαRI triggers NADPH oxidase activation, which produces activated oxygen species, and degranulation in blood-derived eosinophils (54).

5.2.2.3.3 Immunoglobulin E (IgE)

In healthy individuals, IgE is found at an extremely low serum concentration (~0.3 μg/ml), but its biological activity is extremely potent. There are two IgE Fc receptors existing. The low-affinity IgE receptor (CD23, FcεRII), expressed on B cells and macrophages (55,56), and the high-affinity IgE receptor (FcεRI), present on monocytes, DCs and Langerhans cells of allergic patients (57-59). CD23 can be internalized and transports IgE into the antigen-processing pathway, followed by presentation of antigen peptides on the MHC class II molecules, thereby facilitating the presentation of allergens to T cells and resulting in extremely efficient T cell activation. But FcεRI is also able to directly present IgE-bound allergen to specific T cells. It has been shown that in the presence of IgE, antigen uptake and subsequent presentation was increased 100- to 1,000-fold (60). This means, an allergen present at extremely low doses can activate allergen-specific T cells very efficiently via IgE-mediated allergen-presentation, whereas ordinary antigen uptake by APCs via endocytosis or micropinocytosis needs a high antigen concentration. Several publications have shown that strong expression of FcεRI on basophils and mast cells critically depends on the presence of IgE molecules (61-63). Treatment with anti-IgE mAb leads to an enormous decrease of both serum IgE levels, and the levels of FcεRI expressed on peripheral blood basophils and DCs (64-66).

Atopic individuals have a hereditary predisposition to produce IgE antibodies against common environmental antigens and are therefore more susceptible to allergic diseases (67,68). Allergic patients show a strong immune response to innocuous proteins, which should be ignored, because they do not endanger the organism. The cytokines of allergen-specific Th2 cells play a crucial role in the induction of IgE and eosinophilia, factors, which are involved in the immediate hypersensitivity reaction and late-phase responses, respectively. The classical type I IgE-mediated allergic response can be divided into an immediate- and a late-phase response. Allergen-specific crosslinking of IgE bound to the high-affinity IgE receptor on mast cells and basophils leads to the release of inflammatory mediators such as histamine, leukotrienes, and prostaglandins. This results in an immediate hypersensitivity reaction within 5–30 min that lasts for ~1 h. Subsequently, most patients develop a late-phase reaction, which is characterized by the recruitment and persistence of eosinophils, basophils and activated $CD4^+$ T cells at the sites of allergen exposure, and can manifest as nasal or airway obstruction. Generally, late-phase responses last maximal 6–12 h following the immediate reaction and disappear within 24 h (67).

Helminth infections, which are chronic and longlasting, are known to be associated with eosinophil and IgE production and also induce a Th2 immune response. Paradoxically, there is a general observation that the >1 billion people currently infected with helminth parasites, have a lower prevalence of atopy and allergic disorders. Infected individuals usually develop anti-inflammatory networks (IL-10, TGF-β and Tregs) to control the tissue damage that would otherwise result from the continuous challenge of the immune system by foreign antigens, which are permanently released by metybolically active worms (69).

5.2.2.3.4 Immunoglobulin D (IgD)

IgD accounts for about 0.2% of the total serum immunoglobulin and the average serum concentration is 30 μg/ml. IgD, together with IgM, constitutes the BCR of mature B cells. But no biological effector function has been identified for this immunoglobulin isotype (28).

5.2.2.3.5 Immunoglobulin M (IgM)

Plasma cells secrete IgM as a pentamer. In a primary response to an antigen, IgM is the first immunoglobulin class produced, and it is also the first immunoglobulin synthesized by neonates. IgM has a serum concentration of 1.5 mg/ml and constitutes 5%-10% of the total immunoglobulin in serum. In the intercellular tissue fluids, only very low amouts of IgM are found due to its large size. As the pentameric molecule also contains the J chain, IgM can bind to the poly-Ig receptors on secretory cells, which transport it across epithelial linings to mucosal surfaces. IgM activates complement very efficiently, even more than IgG does, because due to the pentameric form more than two Fc regions are brought in close proximity, which is required for complement activation (28).

5.2.2.4 Biological activities of antibodies

5.2.2.4.1 Neutralization

To replicate and proliferate, viruses and intracellular bacteria must enter a cell, and they usually bind to specific molecules on the cell surface for invading. This event can be avoided by pathogen-specific antibodies that constrain the pathogen to dock to its preferred receptor (34). Some antibodies, like IgA, directly bind to microbes in

mucus to prevent colonization of mucosal tissues, and those found in anti-venoms neutralize toxins by binding to them (70).

5.2.2.4.2 Activation of complement

Antibodies that bind to antigens on the surface of microbes attract the first component of the complement cascade (complement protein C1) with their Fc region and activate the "classical" complement system (71). Subsequently, the bacteria can be killed in two ways (34). First, the binding of the antibody and complement fragments on the surface marks the microbe and targets it for phagocytosis. Second, the terminal components of complement cascade form a membrane attack complex to kill the bacterium directly (72).

5.2.2.4.3 Activation of effector cells

Effector cells, which express Fc receptors interact with the bound IgA, IgG, or IgE antibodies (34). This interaction of a particular antibody isotype with the Fc receptor on a particular cell triggers a specific effector function of that cell; for example phagocytes phagocytose, mast cells and neutrophils degranulate, natural killer cells release cytokines such as IFN-γ and cytotoxic molecules. Eosinophils are activated by FcεRI-induced signals and release their granule contents, which finally results in deletion of the pathogen (73).

5.2.2.5 Immunoglobulin diversity

Diversity is a fundamental feature of the adaptive immune system. For a successful recognition and eradication of many different types of microbes, a huge diversity among antibodies is required. This is achieved by variability in the amino acid composition of the antigen-binding sites of an antibody molecule. Consequently, antibodies are able to interact with many different antigens (74). It has been estimated that humans produce about 10 billion different antibodies, each capable of binding a different epitope (75). Although an enormous repertoire of different antibodies can be generated in a single individual, the number of genes available for these proteins is limited. There are several genetic mechanisms existing, like combinatorial and junctional diversity, that enable B cells to generate a diverse pool of antibodies from a relatively small number of antibody genes (76).

Table 2. Immunoglobulin isotypes

Features	**IgG1**	**IgG2**	**IgG3**	**IgG4**	**IgA1**	**IgA2**	**IgE**	**IgM**	**IgD**
Molecular weight	150'000	150'000	150'000	150'000	150'000-600'000	150'000-600'000	190'000	900'000	150'000
% of the total serum immunoglobulin		80%			10-15%			5-10%	0.2%
Normal serum levels (mg/ml)	9	3	1	0.5	3	0.5	0.0003	1.5	0.03
In vivo serum half life (days)	23	23	8	23	6	6	2.5	5	3
Activation of classical complement pathway	+	+/-	++	-	-	-	-	+++	-
Crossing of placenta	+	+/-	+	+	-	-	-	-	-
Present on membrane of mature B cells	-	-	-	-	-	-	-	+	+
Mucosal transport	-	-	-	-	++	++	-	+	-
Induces mast cell degranulation	-	-	-	-	-	-	+	-	-

Activity levels indicated as follows: ++ = high; + = moderate; +/- = minimal; - = none
IgG, IgE and IgD always exist as monomers; IgA can exist as monomer, dimer, trimer, or tetramer. Membrane-bound IgM is a monomer, secreted IgM is a pentamer. Adapted from Goldsby et al., Immunology. Fifth ed. New York: Freeman 2003.

5.3 Peripheral tolerance

Mechanisms like activation-induced cell death, anergy and/or immune response modulation by T regulatory cells (Treg) are essential for the maintenance of peripheral T cell tolerance. The reason why some individuals develop atopic disorders upon exposure to allergens, and simultaneously, others are unaffected, is still not understood. However, it is obvious that a strong interaction of environmental and genetic factors have an influence. An allergic inflammation can be divided into four main processes (Fig. 2): (I) activation of memory/effector T cells and mast cells, eosinophils and basophils, (II) organ-selective homing of those cells, (III) prolonged survival and (IV) reactivation in the allergic organs and initiation of effector functions (77). In an allergic inflammation, T cells are activated by aeroallergens, food antigens, autoantigens or bacterial superantigens (78,79). Simultaneously, the skin, lung- or nose-related chemokine network affects the T cells and they show organ-selective homing (80-82). In the subepithelial tissues, the inflammatory cells show a prolonged survival, interact with resident cells of the organ and are reactivated

(83,84). In atopic dermatitis and asthma, T cells induce IgE production, eosinophil survival and mucus hyperproduction as effector functions (84-86). Furthermore, activated T cells cause bronchial epithelial cell and keratinocyte apoptosis (17-20). Since all those pathological events are dependent on T-cell activation, peripheral T-cell tolerance to allergens can avoid an allergic inflammation.

A critical event in a healthy immune response to allergens as well as in a successful allergen-specific immunotherapy is the development of an allergen-specific effector T cell response towards a Treg cell response (87). Treg cells prevent allergen-specific T cell proliferation and suppress secretion of Th1- and Th2-type cytokines. Effector cells of an allergic inflammation, like mast cells, basophils and eosinophils, can also be suppressed by Treg cells, either directly or indirectly (88).

During SIT, histamine, released in high amounts by mast cells and basophils, contributes to the peripheral tolerance in different ways. It induces production of IL-10 by DCs (89) and Th2 cells (90). In addition, it increases the suppressive activity of TGF-β on T cells (91). All these effects are mediated via HR2, a cAMP-associated G-protein coupled receptor, which is highly expressed on Th2 cells and suppresses IL-4 and IL-13 production and T-cell proliferation (92). Therefore, HR2 probably plays an important role in induction of peripheral tolerance or in active suppression of inflammatory/immune responses.

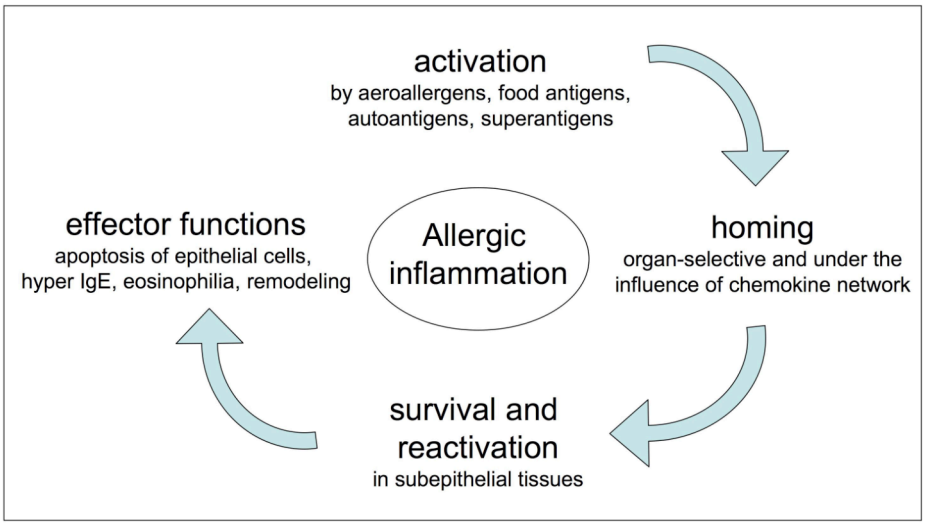

Figure 2. **Allergic inflammation is characterized by four sequential processes.** Several antigens or yet undiscovered factors activate T cells, which subsequently undergo organ-selective homing controlled by organ-related chemokine networks. In

subepithelial tissues, T cells show increased survival due to contacting the extracellular matrix and the cytokines produced in the surroundings, and are continuously stimulated by antigens and superantigens. These activated T cells produce and secrete cytokines, which elicit different effector functions, including apoptosis of the organ cells, hyper-IgE and eosinophilia. Adapted from Taylor et al., 2004, Int Arch Allergy Immunol, 135, 73-82.

5.3.1 T regulatory cells and their cytokines

Immunological tolerance is mediated by central and peripheral mechanisms. Those mechanisms include clonal deletion as well as induction of unresponsiveness in antigen-specific effector T cells. The latter is performed via active suppression by T regulatory cells and is essential for induction of peripheral tolerance to both self and foreign antigens *in vivo*. T cells with suppressive capacity were first described in the early 1970s by Gershon (93,94). Up to now, many distinct types of T regulatory cells have been described (Table 3), which can roughly be divided into natural or inducible Tregs. The three most common subtypes are discussed below.

Table 3. Types of regulatory T cells

Treg cell type	**Suppressor mechanism**
Tr1	IL-10, TGF-β, CTLA-4, PD-1
CD4+CD25+	IL-10, TGF-β, CTLA-4, PD-1, GITR
Th3	TGF-β
CD8+CD25+CD28-	Same as CD4+CD25+
Qa-1-dependent CD8+	Qa-1-specific TCR
CD4-CD8-	Induction of apoptosis
TCR γδ	IL-10, TGF-β

Adapted from Taylor A. et al., Microbes Infect. 2005; 7: 1049-55.

5.3.1.1 Type 1 T regulatory cells (Tr1)

Tr1 cells, also known as adaptive or inducible Tregs, suppress effector T cells in a contact-independent manner by secretion of IL-10 (95) and TGF-β (96,97). Whether an immune respone against common environmental proteins leads to tolerance or allergy depends on a fine balance between allergen-specific Tr1 cells and allergen-

specific Th2 cells (87). It has been observed that in beekeepers, who have a natural tolerance to phospholipase A2 (PLA_2), the major allergen of bee venom, or in patients who underwent SIT, induction of anergy correlates with a considerable increase of IL-10-producing T cells. Neutralisation of endogenous IL-10 fully reconstitutes T-cell proliferation to PLA_2 *in vitro* (98). By using IFN-γ-, IL-4- and IL-10-secreting allergen-specific $CD4^+$ T cells that represent Th1-, Th2- and Tr1-like cells, respectively, it has been found that both healthy and allergic individuals have all three subsets, however, in different proportions. In healthy individuals, the dominant subset specific for common environmental allergens are Tr1 cells, whereas in allergic individuals, allergen-specific IL-4-secreting T cells are the most frequent subset. Therefore, the development of either allergy or a healthy immune response is dependent on the frequency of allergen-specific effector Th2 cells or T regulatory cells (87). Human and mouse naive $CD4^+$ T cells cultured *in vitro* together with vitamin D3 and dexamethasone differentiate into Tr1 cells, which produce only IL-10 (99). Additionally, IL-10, IFN-α or a combination of IL-4 and IL-10 can also lead naive $CD4^+$ T cells to differentiate into Tr1 cells *in vitro*, however, these generated cells produce also moderate amounts of IFN-γ and IL-5 (100-102). Tr1 cells are not anergic and can efficiently proliferate *in vivo* (103). For proliferation *in vitro*, they need IL-2, IL-4, IL-7 and IL-15 (87).

Tr1 cells, and also $CD4^+CD25^+$ Treg cells, constitutively express cytotoxic T lymphocyte antigen-4 (CTLA-4), whereas naive T cells express this molecule only after they have been activated. CTLA-4 is a co-stimulatory receptor of the CD28 family and transduces negative signalling in activated effector T cells, but also contributes to Treg cell-mediated suppression. *In vitro*, blocking of CTLA-4 abrogates the suppressive effect of Treg cells in humans (104). Treatment of young naive mice with monoclonal antibody to CTLA-4 elicits autoimmune disease without reducing the Treg cell number (105). It is suggested that signals via CTLA-4 and TCR together induce the suppressive activity of Treg cells.

A subset of human Tr1 cells expresses programmed death-1 (PD-1), which also belongs to the CD28 family. PD-1 is an immunoreceptor tyrosine-based inhibitory motif–containing receptor expressed upon T cell activation and probably has an inhibitory effect in immune responses, as PD-1–deleted mice develop autoimmune diseases (106). Ligands for PD-1 are PD-ligand (L)1 and PD-L2, members of the B7 family. Binding of PD-L to PD-1 on murine CD4 and CD8 T cells leads to inhibition of proliferation and cytokine production. T cells stimulated with anti-CD3/PD-L1Fc-coated beads show dramatically decreased proliferation and IL-2

production (107).

Tr1 cells which are specific for different antigens arise *in vivo* (108). One allergen-specific Tr1 cell can be found in 1'000 to 20'000 of the total $CD4^+$ T cell population and the antigen-specific suppressive activity of Tr1 cells can be observed already at very low numbers. If the number of cells is very high and sufficient quantities of suppressor signals are around, Tr1 cells also show nonspecific suppression. If the outcome of an immune response is more stimulating or more suppressive might be depending on the T cell which first contacts an APC. In case the first T cell is a Tr1 cell, it may inhibit the maturation of APC, since IL-10 down-regulates the antigen-presenting capacity, such as HLA-DR expression, costimulatory molecules, and several cytokines in DCs and monocytes or macrophages (109). IL-10-secreting T cells seem to be able to regulate the functional state of APCs in a way that these APCs can then promote the generation of more Tr1 cells.

5.3.1.2 CD4+CD25+ T regulatory cells

The natural Treg cells or $CD4^+CD25^+$ T cells characterized by high levels of CD25 (IL-2Rα-chain) and FoxP3 expression originate in the thymus, but they can also be generated in the periphery (110). FoxP3 belongs to the forkhead/winged-helix family of transcription factors and is belived to be the main regulator in the development and function of $CD4^+CD25^+$ Treg cells (110). On average, the frequency of $CD4^+CD25^+$ T cells is approximately 3% in total PBMCs and 5-10% in peripheral $CD4^+$ T cells. They strongly suppress the proliferation of both naive and memory $CD4^+CD25^-$ T cells to alloantigens (111). Their profile of cytokine production is very similar to that of Tr1 cells. But for their suppressive effects, neither IL-10 nor TGF-β seems to be directly required (111). However, suppression requires activation of suppressor T cells by TCR ligands or anti-CD3 antibodies (112), but interestingly, suppression occurs in an antigen-non-specific manner (113). In addition, they express the surface markers CTLA-4 (114) and PD-1, which are important for their suppressive activity, but also glucocorticoid-induced tumour necrosis factor receptor (GITR) (115). GITR negatively regulates $CD4^+CD25^+$ Treg cell suppression, because binding of the soluble GITR ligand to GITR leads to loss of the anergic state of the cell (116). In the presence of IL-2, human $CD4^+CD25^+$ T cells can be expanded *in vitro* and cultured cells retain their suppressive capacities. If $CD4^+CD25^+$ T cells are eliminated, various diseases in genetically susceptible hosts develop spontaneously. The X-linked immune dysregulation, polyendocrinopathy and enteropathy syndrome

(IPEX), in which the FOXP3 gene is mutated and $CD4^+CD25^+$ Treg cells are nonfunctional, is characterized by an allergic phenotype with dermatitis and hyper IgE and an autoimmune phenotype with enteropathy, type I diabetes, thyroiditis, hemolytic anemia and thrombocytopenia (117,118). The role of $CD4^+CD25^+FOXP3^+$ Treg cells in human allergies has been studied in acute atopic dermatitis lesions, where an impaired skin infiltration of $CD4^+CD25^+FOXP3^+$ T cells could be observed (119). In asthmatic patients under glucocorticoid treatment, FOXP3mRNA expression was significantly increased (120).

5.3.1.3 Th3

Th3 cells produce high levels of TGF-β and like Tr1 cells, they are also inducible upon activation with an antigen or anti-CD3 antibody (96,121). Neutralizing antibodies against TGF-β and IL-10 revealed these cytokines to play a crucial role because the disease-protective effects of Th3 cells were abrogated. Furthermore, it has been shown that Th3 cells exert bystander immune suppression *in vitro* (96).

5.3.1.4 Interleukin 10

IL-10, the main cytokine produced by regulatory T cells, has anti-inflammatory features and it significantly inhibits the anti-CD28-induced proliferation. The tyrosine kinase Tyk-2, which is associated to the IL-10 receptor tyrosine phosphorylates SHP-1 upon IL-10 binding. Within minutes, SHP-1 binds to CD28 and ICOS costimulatory receptors and dephosphorylates them. Thereafter, the binding of phosphatidylinositol 3-kinase to CD28 and ICOS no longer occurs, and the downstream signaling is inhibited. Increased proliferation of spleen cells from SHP-1-deficient mice stimulated with CD28 and ICOS could be observed compared to spleen cells of wild-type mice (122). Only T cells stimulated by low numbers of triggered TCRs, which depend on CD28 stimulation, can be inhibited by IL-10 (123). In PBMCs, the proliferative T-cell response to various allergens was inhibited by IL-10, however, the proliferative responses of T cells, stimulated by anti-CD3, was not suppressed (123). IL-10 also indirectly suppresses T-cell responses by inhibiting the antigen-presenting capacity of APCs, like dendritic cells (DCs), Langerhans cells and macrophages. Expression of MHC class II and co-stimulatory molecules such as ICAM-1, CD80 and CD86 is downregulated and the secretion of cytokines and chemokines, which influence T-cell differentiation, proliferation and migration is decreased (109). IL-10 does not only inhibit T cells but also activated monocytes and macrophages (124), however

through different mechanisms since monocytes and macrophages do not express CD28. In monocytes, the suppressor of the cytokine-signalling-3 (SOCS3) gene, which probably inhibits the Stat1-tyrosine phosphorylation induced by IFN, is induced by IL-10 (125).

In mice, administration of IL-10 before allergen treatment leads to unresponsiveness of antigen-specific T-cells, which demonstrates the crucial role of IL-10 in induction/maintenance of peripheral T-cell tolerance (126). Moreover, prevention of graft-versus-host disease by IL-10 and allograft rejection in human leukocyte antigen-mismatched bone-marrow-transplanted severe combined immunodeficient patients provides additional evidence for the importance of this cytokine in the induction and maintenance of peripheral tolerance (127). Similarly, increased endogenous IL-10 production by tumor-reactive human T cells led to a reduced stimulation of these cells which also shows a role for IL-10 in tumor-specific tolerance (128).

In B cells, IL-10 added during the first 3 days of *in vitro* culture has been shown to decrease ε transcript expression and IgE production induced by IL-4. But addition of IL-10 to B cells that already switched to IgE potentiated IgE production. IL-4-induced γ4 transcript expression and IgG4 production was upregulated by IL-10 independent of the time of addition. IL-10 is not a switch factor for IgG4, but it seems to induce the growth and differentiation of cells that have already switched to IgG4 and to synergize the IL-4-induced IgG4 production (129).

5.3.1.5 TGF-β

It has been demonstrated in several studies that $CD4^+CD25^+$ T cells produce high amounts of TGF-β in both mice and humans. Those cells express TGF-β in its active structure on the cell surface (130). Colitis in wild-type mice could be suppressed by $CD4^+CD25^+$ Treg cells from wild-type mice, but not by $CD4^+CD25^+$ cells from a transgenic mouse model with impaired TGF-β signalling specifically in T cells (131). TGF-β not only has suppressor activity, but it has also been shown to be important for the induction of $CD4^+CD25^+$ Treg cells *in vitro* and *in vivo* (132). In TCR-stimulated T cells, TGF-β induces FOXP3 expression, surface expression of CD25, HLA-DR, GITR, CD103 and intracellular CTLA-4 (133). The generated cells do not respond to TCR stimulation, and do also produce TGF-β and IL-10, but they do not synthesize Th1 nor Th2 cytokines. In TGF-β1-/- mice a significantly reduced number of Tregs occurs in the periphery, although the Tregs develop normally in the thymus.

However, the Foxp3 expression is lower and cells are less suppressive, suggesting that TGF-β signaling is essential in maintaining Tregs *in vivo*. Not surprisingly, these mice develop severe autoimmunity (134). In mice, TGF-β toghether with IL-6 induces the differentiation of Th17 cells (135), which have been associated with many inflammatory diseases such as asthma, rheumatoid arthritis, lupus and allograft rejection (136,137). IL-17, the main cytokine produced by Th17 cells, has a proinflammatory function because it recruits neutrophils to inflammatory sites through the induction of granulocyte colony-stimulating factor and IL-8 (138).

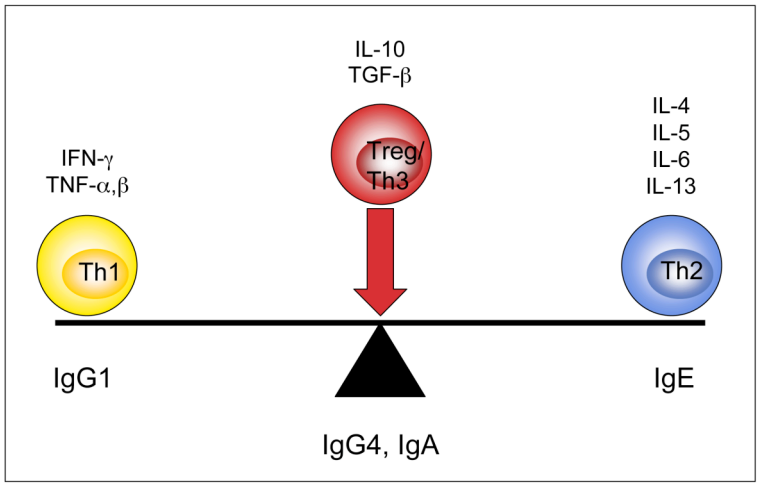

Figure 3. **Treg cells control the immune responses elicited by Th1 or Th2 cells.** IFN-γ released by Th1 cells activates B cells to produce IgG1 antibodies, whereas IL-4 and IL-13 secreted by Th2 cells induces class switch to and production of IgE antibodies. Treg cells however, stimulate B cells to produce/secrete IgG4 and little amounts of IgA. Thus, Treg cells regulate antibody isotypes, which may contribute to the suppression of inflammatory dieseases by inducing IgG4 and by suppressing IgE.

5.3.2 Tregs and the hygiene hypothesis

Atopic disorders have been arising constantly during the past three decades and not only the frequency but also the severity of those disorders has been increasing, particularly in developing countries. Currently, 10-20% of children and 1-3% of adults in industrialized countries have atopic dermatitis (139). The hygiene hypothesis tries to explain the reason for this increase in Th2-associated allergic diseases (140).

Improved public health, the use of antibiotics and vaccines, which leads to a reduction in childhood infections, a decreased exposure to endotoxins, domestic animals or farms and changes in gut flora are all factors which seem to be associated to the enormous increase in allergies (141-144). It is thought that early childhood infections direct the immune response from a Th2 response to an anti-allergic Th1 response (145). However, at the same time as Th2-mediated allergies increased in developed countries, the frequency of Th1-mediated autoimmune diseases also rose. Therefore, the hygiene hypothesis has been adapted to reflect both a deviation in the Th1- and Th2-responses (141). The adaptation of the immune response, which can be observed during parasitic helminth infections, is an exception to this pathogen-induced Th2-Th1 cytokine-shift paradigm, because, individuals with parasitic worm infections have Th2-polarized responses, similar to atopic individuals, however, they normally have reduced allergic responses. Hence, it is possible that the rise in allergic diseases in the developed world is due to a reduction in infections with microbes or parasites. Such infections might induce regulatory cells and cytokines to suppress allergic Th2 or autoimmune Th1 responses (141,146-148). Most parasitic helminths infect humans chronically, it means for years or decades. The parasite gets tolerized through induction of the immune suppression. This chronic downregulation of the immune system evokes a regulatory environment that might protect the individual from allergies (146,149).

5.4 The innate immune system

The first reactions against microbes invading the body are mediated by the innate immunity, which subsequently induce responses of the adaptive immunity. The defense mechanisms of the innate immunity are in place even before infection and responses to infections are very rapid. These mechanisms are specific for structures that are common to groups of related microbes and do not react to noninfectious substances, however, they cannot differentiate tiny distinctions between foreign substances. Furthermore, repeated infections evoke essentially the same responses. The main components of the innate immune system are (I) physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelial surfaces; (II) phagocytic cells (macrophages, neutrophils) and NK cells; (III) proteins of the complement system and other mediators of inflammation; (IV) cytokines that govern many activities of the cells of innate immunity. Receptors of the innate immune system bind conserved pathogenic particles called pathogen-associated

molecular patterns (PAMPs) that are only present on microbial pathogens and not on mammalian cells.

5.4.1 Toll-like receptors

Toll-like receptors (TLRs) are specific for microbial products including bacteria, viruses, fungal and protozoal components and can trigger innate immunity (150,151). They are type I integral membrane glycoproteins and consist of a leucine-rich repeat (LRR) domain flanked by characteristic cysteine-rich motifs in their extracellular regions and a toll/interleukin-1 receptor (TIR) domain in their cytoplasmic region, and these domains are highly conserved. So far, 13 distinct mammalian TLRs have been classified, 10 of which are found in humans. Some TLRs, such as TLR1, TLR2, TLR4, TLR5 and TLR6, are present on the plasma membrane, whereas others, such as TLR3, TLR7, TLR8 and TLR9, are present in the endosome. TLR2 binds zymosan (152), TLR3 oligomerizes in the presence of double-stranded RNA (153), the ligand for TLR4 is LPS, TLR5 binds flagellin (154), TLR7 interacts with single-stranded RNA, TLR8 recognizes single-stranded RNA and imidazoquinoline and TLR9 binds to CpG-containing DNA (155). After binding of the ligand, TLRs dimerize and subsequent conformational change results in recruitment of downstream signalling molecules. However, some TLRs are able to heterodimerize, which leads to an extension of the repertoire of specificities of the TLR system. TLR1/TLR2 bind triacyl lipopeptides, TLR2/TLR6 bind diacyl lipopeptides (156).

With the exception of TLR3 are all TLRs linked to the adaptor molecule myeloid differentiation factor 88 (MyD88) and finally, the nuclear factor (NF)-κB gets activated (157). MyD88 has an N-terminal death domain (DD) and a C-terminal TIR domain. Currently, there are four more cytosolic TIR-containing adapter proteins known, Mal (MyD88-adapter like, also called TIRAP), TRIF (TIR domain-containing adapter-inducing interferon-β, also called TICAM-1), TRAM (TRIF-related adaptor molecule, also called TICAM-2) and SARM (sterile a- and armadillo-motif-containing protein). TLR3 and also TLR4 are coupled to TRIF that leads to the activation of NF-κB and production of type I interferon (IFN). Activation of TLR2 and TLR5 does not induce type I IFN (158-160), however, triggering of TLR7, TLR8 and TLR9 also induces type I IFN production, but through different signalling pathways than via TLR3/4-activation (161,162).

TLR stimulation on DCs, which are responsible for priming naive T cells (163), induces upregulation of MHC and costimulatory molecules, production of IFN-α, and proinflammatory cytokines such as TNF-α, IL-1 and IL-6 as well as the

regulatory cytokines IL-12 and IL-18, which promote Th1 differentiation (164). In MyD88 deficient mice, an increase of Th2 responses with strong IgE production can be observed (165). In mammals, two different DC subsets exist, the myeloid and plasmacytoid DCs. The myeloid DCs (mDC) are similar to monocytes, secrete IL-12, and express TLR2 and TLR4. 8-16 hours after induction of DC maturation, IL-12 production is exhaused and Th2 responses are induced (166). Plasmacytoid DCs (pDC) look like plasma cells, produce high amounts of IFN-α, express TLR7 and TLR9 and induce Th1 differentiation, but like mDCs, when their IFN-α-producing capacity is exhausted, they induce Th2 polarization (167). Moreover, Langenkamp et al. showed that high doses of antigen favor Th1, whereas low doses favor Th2 responses (166).

TGF-β and IL-10 have been shown to be involved in negative regulation of TLR-mediated signalling. TGF-β1 suppresses LPS-mediated responses and inhibits TLR4 expression (168). It also induces MyD88 degradation via ubiquitination (169). IL-10 inhibits pro-inflammatory cytokine production through LPS, and in human DCs, it downregulates IL-12 produced via TLR3- and TLR4-mediated signalling (170).

Triggering of TLR7 and TLR9, which are expressed by B cells and pDCs, needs internalization of the ligand. Both cell subsets are poorly phagocytic, however, they can efficiently internalize BCR- or FcγRIIA-bound molecules. Therefore, B cells bearing a BCR, which recognizes RNA- or DNA-containing molecular complexes, can efficiently transport these complexes to TLR7 and TLR9 in the endosome. pDCs deliver RNA- and DNA-containing IgG immune complexes bound to FcγRIIA to endosomal TLR7 and TLR9. Leadbetter et al. showed that DNA-containing chromatin–IgG complexes could induce autoreactive B cells to produce antibodies against self-IgG (rheumatoid factors) by sequentially engaging BCR and TLR9 (171). In a similar study, RNA-associated autoantigens stimulated autoantibody production by engaging BCR and TLR7 in a sequential fashion and this response was strongly enhanced by IFN-α (172). In mouse models of systemic lupus erythematosus (lpr/lpr mice), it has been shown that defects in the TLR7 and/or TLR9 signaling pathways diminish the disease and that specific antibody production changed in a predictable way. Antibodies to both DNA and RNA were produced in lpr/lpr mice, whereas in MyD88$^{-/-}$ lpr/lpr mice, which are deficient in both TLR7 and TLR9 signaling, antibodies to nucleic acids were absent (172). TLR9-deficient lpr/lpr mice, however, are unable to produce anti-DNA antibodies but anti-RNA antibodies are still present (172,173). Moreover, RNA-specific antibodies are not found in mice that lack TLR7 (174).

5.4.2 TLRs and other innate stimuli in thymus-independent B cell responses

In the absence of T cell help, B cells can be activated by highly repetitive structures which are expressed on the surface of pathogens and which crosslink the BCRs. Splenic marginal zone B cells join B1 B cells in the first 3 days of a primary response to generate an enormous thymus-independent (TI) IgM-antibody response to particulate antigens, such as streptococcus pneumoniae (175). Activated DCs and peritoneal macrophages produce B cell-activating factor (BAFF), which additionally stimulates B cells and thereby sustain this response (176). Another example of TI responses is the IgA response to intestinal flora. Without T cell help or organized lymphoid structures, B1 B cells produce large amounts of intestinal IgA in the presence of intestinal microflora, which is specific for cell wall antigens of commensal bacteria (177).

There are other innate mechanisms existing which are involved in TI antibody responses and are associated to complement activation. BCR signal transduction is facilitated by the complement protein fragment C3d which binds to the microbial surface and simultaneously to its receptor CD21 expressed on B cells and finally results in enhancement of the cellular responses (178). C4-binding protein (C4BP), a protein that binds the complement protein fragments C4b and C3b, is able to engage CD40 on B cells thus imitating a T helper cell (179).

5.4.3 TLRs in thymus-dependent B cell responses

DCs activated by adjuvants or TLR agonists prime T helper cells by presenting the antigen on MHC class II molecules. Subsequently, those helper T cells stimulate B cells. Antibody responses to protein antigens need this interaction of B cells and T helper cells. However, Pasare and Medzhitov studied TLR engagement on B cells in response to protein antigens in mice in which MyD88 deficiency was restricted to B cells (180). Human serum albumin and LPS primed T helper cells were injected into these mice, however, an induction of an optimal antibody response failed to appear. TLR-stimulation on B cells was essential for IgG2 responses and, to a lesser extent, for IgM and IgG1 responses, while IgE responses were TLR-independent. Those results show that direct stimulation of TLRs on B cells increases and modulates the antibody response to a protein antigen even when the TLR agonist is not bound to the BCR (180).

5.4.4 TLRs on human naive and memory B cells

Human naive B cells express only low levels of TLRs unless they are stimulated through the BCR (181,182). In a study using highly purified human naive B cells (183), it has been shown that in addition to BCR stimulation and cognate T cell help, direct triggering of TLRs on responding B cells was required to induce efficient B cell proliferation, isotype switch to IgG and IgA, and differentiation to antibody-secreting cells (184). The coupling that only BCR-stimulated naive B cells express TLRs and therefore can respond to innate signals renders the human immune system highly specific. Human memory B cells constitutively express TLR2, TLR6, TLR7, TLR9 and TLR10, and stimulation of those TLRs leads to proliferation and differentiation to antibody-secreting plasma cells (181,182,185).

5.4.5 TLRs and class switch recombination

There is some evidence that TLR stimulation on B cells influences isotype switching. In mice, class switching to the pathogenetic IgG2a and IgG2b autoantibodies requires TLR9 and MyD88, however, development of IgM autoantibodies does not (186). Moreover, TLR9-triggering, but not TLR4-triggering, upregulates T-bet expression in B cells and induces IgG2a while it decreases IgG1 and IgE production (187,188). The CpG-induced inhibition of IgG1 and IgE is T-bet-independent and might be mediated by induction of B-cell lymphoma protein 6 (Bcl-6) or inhibitor of DNA binding protein 2 (Id2) (189). In human B cells, activation of TLR9 by CpG-DNA initiates germline immunoglobulin heavy chain constant region Cg1, Cg2 and Cg3 gene transcription, in association with IL-10 (190). In addition to directly acting on B cells, TLR agonists can also indirectly influence B cell differentiation and isotype switching via activation of DCs. Activated pDCs release IFN-α and IL-6, which are responsible for the differentiation of plasma cells (191) and induce expression of BAFF and a proliferation-inducing ligand (APRIL) on conventional DCs. BAFF and APRIL trigger CD40-independent class switch recombination very efficiently (192). mDCs triggered by synergizing TLR stimuli (193) produce IL-12, which subsequentially activates human naive B cells to differentiate into IgM producing plasma cells (194). In a recent study, retinoic acid synergized with gut-associated lymphoid tissue (GALT)-DC-derived IL-6 or IL-5 to induce IgA secretion (195), however, the role of TLRs in this system remains to be elucidated. Originally, it has been tought that isotype switch was restricted to germinal centers of secondary lymphoid organs, but in several studies it has been shown to take place also outside

organized lymphoid tissues. In mice, IgM^+ lymphocytes in the gut lamina propria switch *in situ* to IgA (196). In humans, IgE switching has been observed in the nasal mucosa of allergic rhinitis patients (197). In another study, activation of TLR3 on reticular epithelial cells of human tonsils led to the production of BAFF and IL-10, which enhance class switching and IgG and IgA secretion in naive B cells. This response was amplified by thymic stromal lymphopoietin (TSLP) but inhibited by secretory leukocyte protease inhibitor (SLPI) (198).

5.4.6 TLRs and regulatory T cells

TLRs are also expressed on regulatory T cells. Wang et al. demonstrated that a direct stimulation of TLR8 on human Treg cells can reverse Treg cell function (199). TLR8 ligand-stimulated Treg cells were transfered into tumor-bearing mice resulting in a dramatic enhancement of the anti-tumor immunity. Another study showed that in $TLR2^{-/-}$ mice, the number of $CD4^+CD25^+$ Treg cells is significantly reduced compared to wildtype mice (200). Surprisingly, combined stimulation of TCR and TLR2 on Tregs enhanced Treg proliferation *in vitro* and *in vivo* and resulted in a temporal loss of the suppressive Treg phenotype *in vitro* (200). Hence, TLR-mediated regulation of Treg might be linked to the onset and severity of allergic diseases.

5.4.7 TLRs in allergic disease

The enhanced prevalence of allergic diseases in the industrialized countries has been explained by the hygiene hypothesis. Bacterial and viral infections during early childhood are believed to direct the maturing immune system towards Th1, which counterregulates pro-allergic Th2 responses. Increased hygiene and a reduced microbial environment result in low stimulation of TLRs and subsequently in weak Th1 imprinting. This leads to unrestricted Th2 responses that presumably provoke allergies (140). This hypothesis is supported by the observation that viral infections such as hepatitis A, measles and tuberculosis are negatively associated with the incidence of allergic diseases (201). Children, who are growing up in a farm and have a decreased risk of developing allergies, have an enhanced expression of TLR2mRNA. Therefore, TLR2 might modulates the development of allergic diseases (202). Furthermore, it has been identified that polymorphisms in the TLR2 gene are associated to the susceptibility to develop allergic diseases (203,204). In an epidemiological study, also the TLR4 signaling pathway has been shown to play a role in the development of allergies. In homes of allergen-sensitized infants, house

dust endotoxin levels were lower compared to houses of non-sensitized infants. Therefore, the idea arose, that indoor exposure may protect against allergen sensitization (205). LPS exposure and atopy rates in farmers' family members correlated inversely supporting this hypothesis (206). Moreover, polymorphisms of CD14, a LPS coreceptor for TLR4, influenced the severity of atopy (207). The knowledge about the association of other TLRs and the development and severity of allergic diseases is little, but for intervention and treatment of allergic disease, several TLRs have been used as targets.

5.5 Allergen-specific immunotherapy

5.5.1 Conventional immunotherapy

More than 25% of the population in industrialized countries shows IgE-mediated hypersensitivity, which is the most common form of allergy (208). Various environmental proteins, known as allergens, elicit an allergic response. Allergic rhinitis, allergic asthma, food allergy, allergic skin inflammation, ocular allergy and anaphylaxis are clinical manifestations of allergic responses. Allergic inflammation and reactions to allergens can be either local, like in allergic rhinitis or allergic asthma, or systemic, like in anaphylaxis.

The etiology of allergic diseases is very complex and several factors, including genetic susceptibility, route of exposure, dose and structural characteristics of the allergen have an impact (209). Antihistamines, antileukotrienes, β2-adrenergic receptor antagonists and corticosteroids can temporarily suppress the mediators and immune cells and thus alleviate the symptoms of IgE-mediated allergic reactions (92,210,211). Allergen-specific immunotherapy (SIT) is the only treatment so far which leads to long-term recovery and specifically reestablishes a normal immunity against allergens. This treatment involves repeated, mostly subcutaneous, administration of the allergen, and has been applied most efficiently to insect venom allergy and allergic rhinitis (212-214). An increase of IgG4 as well as a decrease in the number of mast cells and eosinophils, which are responsible for the release of mediators (88,215-217) can be observed in a successful allergen-SIT. Furthermore, IL-4 and IL-5 production by $CD4^+$ T cells was decreased during allergen-SIT (214,218). In SIT to bee venom, wasp venom, grass pollen and house dust mite (HDM), a change from a Th2 cytokine pattern towards a Th1 phenotype with increased IFN-γ production was observed (219,220). Importantly, the induction of a

tolerant state in peripheral T cells seems to be a crucial step during allergen-SIT (98,221,222). Autocrine action of IL-10 and TGF-β increasingly produced by the antigen-specific T cells leads to T cell tolerance (98,221). These cells express CD4 and CD25 and could be both, inducible Tr1 cells, which have upregulated CD25 or naturally occurring $CD4^+CD25^+$ Treg cells that produce suppressive cytokines (223). $CD4^+CD25^+$ Treg cells from atopic donors have been shown to have a reduced capability to suppress the proliferation of $CD4^+CD25^-$ T cells (224). Thus, upregulation of $CD4^+CD25^+$ Treg cells probably plays an important role in allergen-SIT. Depending on the cytokine that is present in the tissue microenvironment, tolerized T cells can be reactivated and start to produce either Th1 or Th2 cytokines. Therefore, allergen-SIT might be directed towards successful or unsuccessful treatment (225). During the early phase of SIT, serum levels of both specific IgG4 and IgE increased. However, the antigen-specific IgG4 increase was stronger and thus, the ratio of specific IgE to IgG4 decreased by 10- to 100-fold (225). The development of such a specific isotype ratio could be observed in SIT against various allergies. Furthermore, during SIT, increasing amounts of IL-10 are produced. IL-10 has been shown to suppress both total- and allergenspecific IgE, but to increase IgG4 production (98,129,226). In healthy individuals, response to Der p1 showed enhanced specific IgA and IgG4, low amounts of IgG1 and nearly undetectable IgE antibodies in serum (222). Although antigen-specific IgE levels did not decrease significantly after 70 days of treatment during HDM SIT, antigen-specific IgA, IgG1 and IgG4 showed a significant rise (222). Additionally, the increase of specific IgA and IgG4 in serum coincided with elevated concentrations of TGF-β and IL-10, respectively (98,222,227).

The fundamental mechanisms occurring during allergen-SIT are continuously being clarified. The very early effects comprise mast cell and basophil desensitization, intermediate effects are characterized by changes in allergen-specific T cells and late effects imply B cells (228), IgE as well as mast cells, basophils and eosinophils. Several years of SIT are needed to observe a decrease in IgE antibody levels and IgE-mediated skin sensitivity, but interestingly, most allergic individuals show a protective response against bee-stings already at an early stage of bee venom-SIT. One explanation for this is that effector cells of allergic inflammation, such as mast cells, basophils and eosinophils are dependent on T cell cytokines for priming, activity and survival (229,230), which are not sufficiently available because of suppressed Th2 and Th1 cells. The SIT efficiently enhances the thresholds for mast cell and basophil activation and simultaneously, reduces histamine release mediated by IgE (231,232). Moreover, IL-10 reduces the secretion

of proinflammatory cytokines from mast cells (233), down-regulates eosinophil activity and prevents IL-5 production by human resting Th0 and Th2 cells (234). Besides, it inhibits endogenous granulocyte monocyte colony-stimulating factor (GM-CSF) production and CD40 expression by activated eosinophils and induces eosinophil cell death (235).

5.5.2 Current developments in specific immunotherapy

Peptide immunotherapy (PIT) is another attractive approach for safe SIT. Such allergen peptides are short and consist of either original sequences or have some altered amino acid substitutions, but they still contain the linear T cell epitopes, however, not the three-dimensional B cell epitopes. Therefore, IgE cross-linking, which can induce anaphylaxis, is inhibited (236). Several years ago, application of PIT using exact T cell epitope peptides from the major bee venom allergen PLA_2 was very successful (237). During this study, development of specific T cell tolerance and a decrease in the specific IgE:IgG4 ratio could be observed. Various allergen-peptides are now available for PIT, including the latex allergen Hev b 6.01, the cat allergen Fel d 1, the house dust mite allergen Der p 1 and birch pollen allergen Bet v 1 (238-241). Patients undergoing PIT usually tolerate this treatment very well, because peptide vaccination does not induce IgE responses. In contrast, Th2 responses are reduced and simultaneously, IL-10 secretion is enhanced, suggesting an increase of Tr1 cells. One disadvantage of PIT, however, is the limited applicability, because due to the very individual MHC class II-mediated antigen presentation, not all patients are able to respond to the same peptides.

The usage of recombinant hybrid allergen proteins could be another useful method for SIT (242,243). In a single molecule, several T cell epitopes are linked together. Therefore, the immunogenicity of the components is increased compared to separate molecules. In human skin-prick tests, a hybrid allergen protein consisting of three major bee venom allergens showed to be extremely hypoallergenic, and in a mouse model, it could also prevent allergic sensitization to bee venom (242).
Vaccination with allergen-encoding DNA has been proposed as a further strategy for SIT (244,245). This method enhanced allergen-specific Th1 cell responses and, when administered prophylactically in mouse models, prevented allergic sensitization. The disadvantage however, is that transfected cells produce allergen systemically, thereby enhancing the potential of anaphylaxis (246). However DNA encoding hypoallergenic allergens could reduce allergenicity.

Sublingual administration of allergen is another approach for SIT and

immediate swallowing of allergen as a therapy has been used since 1927. During sublingual immuno therapy (SLIT) the allergen is captured by Langerhans cells within the oral mucosa. Thereafter, these cells mature and migrate to proximal draining lymph nodes, where the synthesis of blocking IgG antibodies and the induction of Treg cells is started (247,248). Sublingual immunotherapy (SLIT) is clinically efficacious, but so far, the treatment effect is only about half of that achieved with subcutaneous SIT (249).

5.6 Histamine in allergic inflammation

5.6.1 Histamine

Histamine, 2-(4-imodazole)-ethylamine, is mainly produced by mast cells and basophils, gastric enterochromaffin-like cells, platelets and histaminergic neurons. But also in other cells of the immune system like monocytes/macrophages, DCs, neutrophils and T and B cells, high histidine decarboxylase activity is observed. However, although these cells do not store histamine, they can produce high amounts of this mediator, which is secreted immediately after synthesis (250-253). Released from mast cells and basophils, histamine triggers acute symptoms like rhinitis, bronchospasm, cramping, diarrhea or cutaneous wheal and flare responses due to its very rapid activity on vascular endothelium and bronchial and smooth muscle cells. However, it influences both normal human physiology as well as various pathologies. It is involved in the cell differentiation and proliferation, embryonic development, hematopoiesis, wound healing and regeneration (254-258), but has also an influence in the central nervous system on cognition and memory, regulation of the sleep/wake cycle, energy and endocrine homeostasis (259-261). Furthermore, histamine exhibits all the properties of a classical leucocyte chemoattractant, including actin polymerization, mobilization of intracellular calcium, alteration in cell shape, and up-regulation of adhesion molecule expression.

5.6.2 Histamine receptors

So far, there are four types of histamine receptors (HRs) known that belong to the G-protein-coupled receptor family: HR1, HR2, HR3 and HR4 (Table 4). They are seven transmembrane proteins that use G proteins and intracellular second messengers to transduce extracellular signals (262,263). The four HRs have different K_i values

ranging from 2-10 μM for the HR1 and HR2 to 5-10 nM for the HR3 and HR4 (264,265). Triggering of the $G_{q/11}$-coupled HR1 leads to many of the symptoms of allergic disease in the skin, nose, and lower airways.

HR1 and HR2 are expressed on numerous cells, including airway and vascular smooth muscle cells, hepatocytes, chondrocytes, nerve cells, endothelial cells, DCs, monocytes, neutrophils, T and B cells (266-268). Histamine enhances the secretion of proinflammatory cytokines such as IL-1α, IL-1β, IL-6 as well as chemokines such as regulated activation (RANTES) or IL-8, in several cell types and local tissues and thus contributes to the progression of allergic inflammation (269-272). HR1 and HR2 are also expressed on endothelial cells, and via HR1, histamine infusion leads to increased expression of adhesion molecules such as intracellular adhesion molecule (ICAM-1), vascular cellular adhesion molecule (VCAM-1) and P-selectin (273-275). The expression of histamine receptors on endothelial cells is regulated by the ligand itself (276).

Granulocyte accumulation in tissues is regulated by histamine in distinct ways. Allergen-induced recruitment of eosinophils in the skin, nose and airways can be inhibited by HR1 antagonists (277). Depending on the concentration of histamine, eosinophil migration varies. High concentrations inhibit eosinophil chemotaxis via HR2, low concentrations enhance eosinophil chemotaxis via HR1 (278). In addition, histamine inhibits neutrophil chemotaxis, activation, superoxide formation and degranulation due to HR2-triggering (279).

HR3 is expressed in the central and peripheral nervous systems where it functions as a pre-synaptic receptor controlling the release of histamine and other neurotransmitters. The control of mast cells by histamine acting on HR3 involves neuropeptide-containing nerves and might be related to a local neuron-mast cell feedback loop controlling neurogenic inflammation (280). In case of dysregulation of this feedback loop, excessive inflammatory responses appear which suggests that HR4 agonists may represent a novel therapeutic approach.

HR4 is coupled to protein $G_{i/o}$, inhibiting forskolin-induced cAMP formation like HR3 (263). HR4 is highly expressed in the bone marrow and on peripheral hematopoietic cells, neutrophils, eosinophils and T cells. Moderate expression of HR4 is found in spleen, thymus, lung, small intestine, colon, and heart (263). Both basophils and mast cells express HR4mRNA (281). Recruitment of inflammatory cells, particularly eosinophils and mast cells, at sites of allergic inflammation is promoted by stimulation of HR4 (282). The presently available HR3 ligands can also be bound by the HR4, due to the high homology between the two receptors (263). Recently, it has been shown that the HR1/HR2 antagonist doxepin and the HR1

antagonist cinnarizine and promethazine have high affinity to the HR4. Administration of HR4 antagonists could eliminate HR4-mediated chronic inflammatory effects of histamine, and treatments with combinations of HR4 and HR1 antagonists may be very useful.

Table 4. Histamine receptors

	H1 receptor	H2 receptor	H3 receptor	H4 receptor
Expression	Nerve cells, airway and vascular smooth muscles, hepatocytes, chondrocyes, endothelial cells, neutrophils, eosinophils, monocytes, DC, T and B cells	Nerve cells, airway and vascular smooth muscles, hepatocytes, chondrocyes, endothelial cells, neutrophils, eosinophils, monocytes, DC, T and B cells	Histaminergic neurons, eosinophils, DC, monocytes; low expression in peripheral tissues	High expression on bone marrow and peripheral hematopoietic cells, eosinophils, neutrophils, DC, T cells, basophils, mast cells; Low expression in nerve cells, hepatocytes peripheral tissues, spleen, thymus, lung, small intestine, colon and heart
Intracellular signals	Ca^{2+}, cGMP, phospholipase D, phospholipase A2, NFκB	Adenylate cyclase, cAMP, c-Fos, c-Jun, PKC, p70S6K	Ca^{2+}, MAP kinase, inhibition of cAMP	Ca^{2+}, inhibition of cAMP
Activities of histamine in allergic inflammation and immune modulation	Release of histamine and other mediators ↑ Adhesion molecule epression ↑ Chemotaxis of eosinophils and neutrophils ↑ Antigen-presenting capacitiy ↑ Co-stimulatory activity on B cells ↑ Cellular (Th1) immunity, IFN-γ, autoimmunity ↑ Humoral immunity ↓ IgE production ↓	IL-10 production ↑ Development of Th2 and tolerance-inducing DCs ↑ Humoral immunity ↑ Suppression of Th2 cells and cytokines ↑ Chemotaxis of eosinophils and neutrophils ↓ Production of IL-12 by DCs ↓ Cellular immunity ↓ Plays a role in allergy, autoimmunity, graft rejection	Proinflammatory activity and antigen-presenting capacitiy ↑ Involved in control of neurogenic inflammation by neuron-mast cell feedback loop	Eosinophil chemotaxis ↑ IL-16 production ↑ (HR2 is also involved)
Antagonists	> 40; including diphenhydramine, cetirizine, desloratadine, fexofenadine, loratadine	Cimetidine, famotidine, nizatidine, ranitidine	None	None

Adapted from Akdis C.A. et al., European Journal of Pharmacology. 2006; 533: 69-76.

5.6.3 Immunoregulatory effects of histamine on APCs

Immature and mature DCs express all four HRs (283-285) and during the differentiation of monocytes to mDCs, activation of HR1 and HR3 induces antigen-presentation capacity and proinflammatory cytokine production as well as Th1 priming activity. HR2, however, suppresses the antigen-presentation capacity, enhances production of IL-10 and induces IL-10-secreting T cells (Fig. 4) (89,286,287).

In monocytes that have been activated by TLR triggering, histamine decreases the production of proinflammatory IL-1-like activity, TNF-α, IL-12 and IL-18 but simultaneously, enhances IL-10 secretion via HR2 stimulation (272,287-290). Moreover, on human monocytes, histamine down-regulates CD14 expression through HR2 (291). In immature DCs, histamine induces intracellular Ca^{2+} flux, actin polymerization, and chemotaxis due to stimulation of HR1 and HR3, but when DCs mature, these responses disappear and histamine induces intracellular cAMP levels and IL-10 secretion, while IL-12 production is suppressed via HR2 (89). Human mDCs express both HR1 and HR2 and can induce CD86 expression by histamine, but surprisingly, human epidermal Langerhans cells (LC) express neither HR1 nor HR2 receptors, probably because of the effect of TGF-β (292), which has been identified as a mandatory factor for the development of LCs, but not for that of interstitial/dermal DCs (293). Soga et al. showed that histamine prevents monocytic apoptosis via HR2 and cAMP pathway and that this phenomenon results partially from the histamine-induced endogenous production of IL-10 (294). In chronic allergic disorder like asthma or atopic dermatitis, HR2 signals prolong the lifespan of monocytes and the infiltration to the site of inflammation.

HR1 antagonists inhibit allergen-induced inflammatory cell accumulation in the skin, nose and airways. A possible mechanism for HR1 antagonists might be the downregulation of NF-κB, a potent transcription factor, which plays a role in initiating inflammation by inducing proinflammatory cytokines (295). Furthermore, HR1 antagonists decreased the amounts of proinflammatory cytokines, mediators (e.g. histamine, leukotrienes, prostaglandin), cells (e.g. neutrophils, eosinophils), cell adhesion molecules (e.g. intercellular and vascular cell adhesion molecules) in the lavage fluid of asthmatic patients. In a murine model of allergen-induced airway inflammation and hyperresponsiveness, fexofenadine diminished a Th2-like response by decreasing secretion of IL-4, IL-5, allergen-specific IgE and eosinophilia in lung tissue and bronchoalveolar lavage fluid (296). Therefore, HR1 antagonists, which have weaker anti-inflammatory effects than corticosteroids, may modulate the

balance between Th1, Th2 and Tr1 cells and suppress the accumulation of inflammatory cells.

5.6.4 Effects of histamine on Th1 and Th2 cells

Th1 cells express HR1 predominantly, but not exclusively, whereas Th2 cells show higher expression of HR2. Due to different intracellular signaling pathways, activation of HR1 elicits Th1 responses, while triggering of HR2 decreases both Th1- and Th2-responses (92). In mice, deletion of HR1 leads to decreased IFN-γ production and to enhanced secretion of IL-4 and IL-13. Deletion of HR2 results in upregulation of both Th1 and Th2 cytokines. In another study, it has been shown that histamine induced IL-10 secretion via HR2 (90). Increased IL-10 production by histamine in both DCs and T cells might be an important regulatory mechanism for the control of inflammation (Fig. 4).

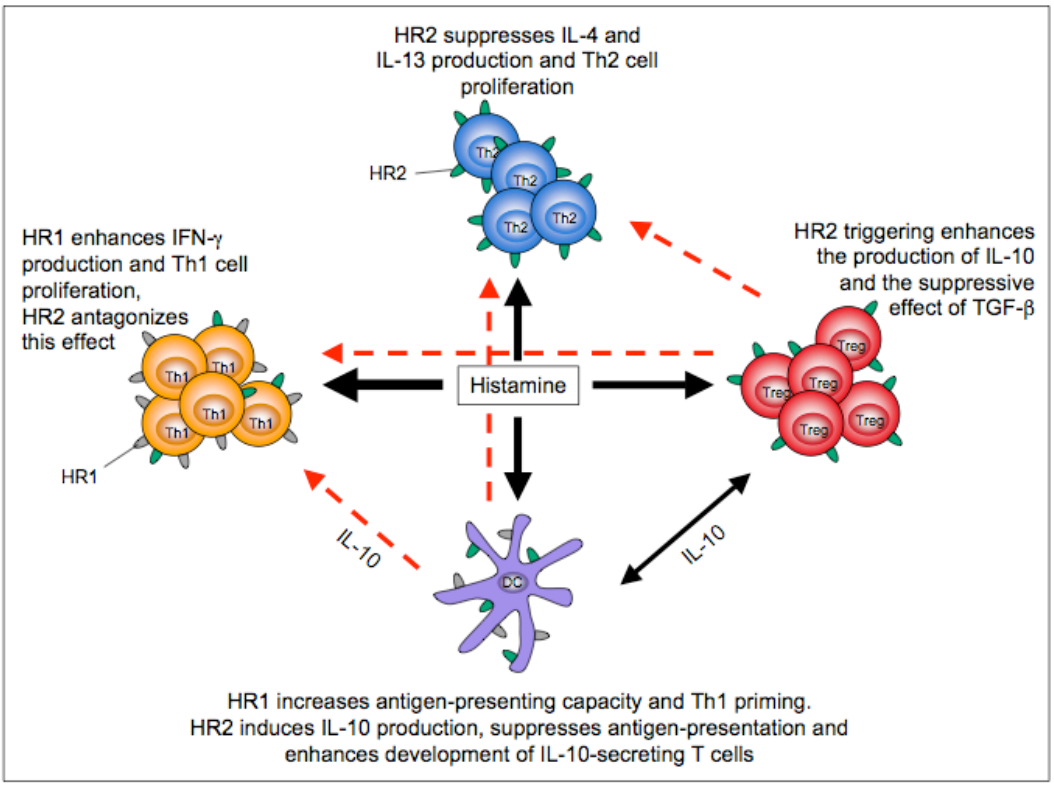

Figure 4. **Histamine influences the inflammatory functions of T cells and APCs in lymphatic organs and subepithelial tissues.** DCs express all four HRs, whereas Th1 cells express predominantly HR1. Stimulation of HR1 on Th1 cells induces their proliferation and IFN-γ production. Th2 cells show a higher expression

of HR2. Histamine released from effector cells suppresses both Th1 and Th2 responses and induces IL-10 production in DCs, Treg and Th2 cells via HR2. IL-10-secreting DCs contribute to Treg cell generation. Furthermore, HR2 potentiates the effect of TGF-β (solid black arrows: activation; dotted red arrows: suppression). Adapted from Jutel et al., 2006, Allergy, 61, 796-807.

5.6.5 Role of histamine in T cell tolerance to allergens

During SIT, the peripheral tolerance is also influenced by histamine in several ways. Histamine induces the IL-10 production by DCs (89) and by Th2 cells (90). In addition, it increases the suppressive activity of TGF-β on T cells (91). These effects are all mediated via activation of HR2 (92), which seems to represent an essential receptor that plays an important role in induction of peripheral tolerance or active suppression of inflammatory immune responses.

Because of their ability to modify immune responses, HR2 antagonists have attracted great attention. Cimetidine shows a stimulatory effect on the immune system, probably by binding to the receptors on the T cell subsets and thereby preventing HR2-induced immune suppression (297). A beneficial effect of cimetidine has been observed when administered as supplement to surgical excision in patients with colorectal, gastric, breast or ovarian cancers, and melanoma (298).

5.6.6 Regulation of antibody isotypes by histamine

HR1 signaling seems also to play an important role in responses triggered from BCRs. In mice, the proliferation of B cells stimulated with anti-IgM is enhanced by histamine, whereas in HR1-deleted mice, antibody production against a T cell-independent antigen TNP-Ficoll is reduced (280). However, in response to T cell-dependent antigens, such as ovalbumin, HR1-deleted mice produced high OVA-specific IgG1 and IgE compared to wild-type mice (280). In HR2-deleted mice, decreased serum levels of OVA-specific IgE was observed compared to wild-type and HR1-deleted mice. Although the IL-4 and IL-13 production by T cells in HR2-deficient mice increased, OVA-specific IgE was suppressed due to highly enhanced IFN-γ. Thus these findings suggest that HR1 and the related Th1 response play an important role in the suppression of humoral immune responses.

6 Results

6.1 Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors

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Key words: B cells, immunoglobulins, regulatory T cells, IL-10, toll-like receptors

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Background: Allergic diseases are characterized by the activation of the immune system and formation of immunoglobulin (Ig) E antibodies against normally innocuous environmental antigens, whereas IgG4 and IgA represent non-inflammatory and blocking antibody isotypes. The T helper 2 (Th2) cells induce and T regulatory (Treg) cells suppress several features of allergic inflammation. Our aim was to investigate the role of allergen-specific T regulatory type 1 (Tr1) cells and $CD4^+CD25^+$ Treg cells and toll-like receptors (TLRs) on IgE, IgG4 and IgA production.

Methods: Germline or productive Ig-transcripts are determined by real-time RT-PCR, secreted Igs are measured by ELISA and the frequency of Ig-producing plasma cells is investigated by ELISPOT. Circulating $CD4^+CD25^+$ Treg cells and allergen-specific Tr1 cells are used.

Results: Both allergen-specific, IL-10-secreting Tr1 cells and $CD4^+CD25^+$ Treg cells from healthy individuals induced IgG4 and suppressed IgE production in peripheral blood mononuclear cells (PBMC) and purified B cell cultures. In contrast, induction of IgA production is independent of T cell help and the role of Tr1 or Treg cells is very limited, whereas it was highly induced by direct B cell activation via TLR7 and 9.

Conclusions: These data suggest that T regulatory cells may contribute to the suppression of allergic diseases by suppression of IgE and induction of IgG4, whereas IgA production is enhanced by B cell activation via TLR7 and TLR9.

Key words: B cells, immunoglobulins, regulatory T cells, IL-10, toll-like receptors

Introduction

The physiopathology of allergic immune responses is complex and has been shown to be regulated by multiple factors. Hallmark of the allergic immune response is the development of allergen-specific Th2 cells and their cytokines IL-4 and IL-13, which are responsible for IgE and IgG4 production by B cells (299). The type and amount of antibodies produced vary according to the type of allergen/antigen, involvement of T cells, prior history of antigen exposure, and anatomic site. Class switch recombination in B cells requires two signals. The first is delivered by cytokines, which target specific C_H genes for transcription, the second is delivered in the case of T-dependent antigens by interaction of CD40 on B cells with its ligand CD40L on activated T cells (35).

Patients suffering from asthma, allergic rhinitis and atopic dermatitis, have elevated serum levels of both total IgE and of IgE that is specific for the allergens that drive these diseases (300,301). The induction of a tolerant state in peripheral T cells represents an essential step in healthy immune response to allergens in sensitized individuals (87). Peripheral T cell tolerance observed in healthy individuals and during allergen-specific immunotherapy is characterized mainly by generation of allergen-specific Tr1 cells and increase in allergen-specific suppressive capacity of whole $CD4^+CD25^+$ T cells (98,222,224). This is accompanied by a significant increase in allergen-specific IgG4 and in some cases IgA and a decrease in IgE in the late stage of the treatment (222,301,302). Moreover, it has been shown that IL-10 decreased IL-4-induced IgE switching but augments IL-4-induced $\gamma 4$ transcript expression and IgG4 production (129).

Along with CD4 and CD25 expression, Treg cells are also associated with the transcription factor Foxp3 (303). Various autoimmune diseases such as arthritis, diabetes and X-linked immune dysregulation, polyendocrinopathy and enteropathy syndrome (IPEX) with hyper-IgE may develop spontaneously when $CD4^+CD25^+$ Treg cells are eliminated, suggesting a role for the control of IgE production (304). Immunoglobulin isotype switch, which was originally thought to be restricted to germinal centers of secondary lymphoid organs, can occur outside organized lymphoid tissues. IgE switch has been shown to take place in the nasal and bronchial mucosa of allergic rhinitis and asthma patients (197,305,306).

Human memory B cells express TLR2, TLR6, TLR7, TLR9 and TLR10, but not TLR3 and TLR4 (181,182). TLR7 and TLR9 recognize nucleic acids of microbial as well as of endogenous origin (307) and they are both present in endosomal compartments. As B cells are poorly phagocytic, but can efficiently internalize

molecules that bind to the B-cell receptor (BCR), they can target RNA- or DNA-containing molecular complexes, which are capable of binding to the BCR, to TLR7 or TLR9 in the endosomal compartment. Consequently, TLR agonists can directly activate human memory, but not naive B cells and it has been suggested that this selective responsiveness may be a mechanism to maintain serological memory (307).

In the present study, we demonstrate that T regulatory cells regulate antibody isotypes, which may contribute to the suppression of inflammatory diseases by the induction of IgG4 and by the suppression of IgE. However, production of IgA is not influenced by regulatory T cells, but is strongly dependent on the innate immune system, particularly TLR7 and TLR9 activation of B cells.

Methods

Medium, antigen, cytokines and TLR-agonists

The medium used for 5-day-cultures was RPMI 1640 supplemented as previously described (87). The medium used for 12-day-cultures was additionally supplemented by 40 μg/ml of transferrin and 4 μg/ml of insulin (both from Sigma, Buchs, Switzerland). Recombinant Dermatophagoides pteronyssinus group 1 (Der p 1) of house dust mite (Allergopharma, Reinbek, Germany) and phospholipase A2 of bee venom (PLA, Sigma Chem. Co.) were used as allergens. They did not contain detectable amounts of LPS and were 99% pure. Recombinant human IL-4 was from Novartis, Basel, Switzerland, IL-10 from PeproTech, London, UK and anti-human IL-10 receptor from ATCC, Middlesex, UK. Soluble CD40L ligand (sCD40L) was produced from the transfected cell line 8-40-1, originally generated by Dr. P. Lane (Birmingham, UK) and cultured for 3 d in CG medium (Vitromex GmbH, Vilshofen, Germany) and standardized according to highest IgE inducing capacity after 12 d culture of PBMC co-stimulated with 50 ng/ml of IL-4 (308). Supernatants obtained from the corresponding untransfected cell line J558L (kindly provided by Dr. M. Reth, University of Freiburg, Freiburg, Germany) were used as control.

Following TLR agonists were used: Pam3-Cys-Ser-$(Lys)_4$ for TLR2 (Calbiochem AG, Laufelfingen, Switzerland), poly(I:C) for TLR3 (Amersham Biosciences, Lausanne, Switzerland), E. Coli derived lipopolysaccharide for TLR4 (Sigma, Buchs, Switzerland), flagellin for TLR5 (Calbiochem AG), 3M-001 for TLR7 (3M Pharmaceuticals, Minneapolis, USA), 3M-002 for TLR8 (3M Pharmaceuticals), CpG2006 for TLR9 (Microsynth GmbH, Balgach, Switzerland).

Purification of B cells, Tr1 and $CD4^+CD25^+$ Treg cells

PBMC were isolated by Ficoll (Seromed, Vienna, Austria) density gradient centrifugation from peripheral venous blood of healthy donors. B cells were isolated by depleting non-B cells by using a cocktail of biotin-conjugated antibodies against CD2, CD14, CD16, CD36, CD43 and CD235a and anti-biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of B cells was more than 95%.

For the isolation of IL-10-secreting cells, PBMC were stimulated with 0.3 μmol/L antigen in 5 ml of medium in six-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) (87). After 12 h of stimulation in humidified 5% CO_2, cells were harvested and labeled with anti–IL-10/CD45 Ab–Ab conjugates (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were diluted with 37°C warm medium to a

final concentration of 10^6 cells/ml and allowed to secrete and capture IL-10. After capturing the secreted cytokine on their surface, cells were stained with PE-conjugated anti–IL-10 mAb. The cells were washed and resuspended in PBS, containing 0.5% bovine serum albumin (BSA) and 2 mmol/L EDTA, and magnetically labeled with anti-PE microbeads. After another washing step, labeled cells were purified (AutoMacs, Miltenyi Biotec, Bergisch Gladbach, Germany).

Purification of $CD4^+CD25^+$ Treg cells was performed in a two-step procedure. First, non-$CD4^+$ cells were depleted with a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and glycophorin A and anti-biotin microbeads. In the second step, $CD4^+CD25^+$ T cells were directly labeled with anti-CD25 microbeads and isolated by positive selection from the pre-enriched $CD4^+$ T cell fraction. The cells were counterstained by FITC-labeled anti-CD4 and PE-labeled anti-CD25 mAb (Beckman Coulter, Fullerton, CA, USA) and analyzed in a flow cytometer (Epics XL; Beckman Coulter). The purity of $CD4^+CD25^+$ Treg cells was between 92.5% and 96.9%. High Foxp3 expression in $CD4^+CD25^+$ cells, purified by this method, has been previously reported (119).

Cell cultures

PBMC or pure B cells from non-allergic and healthy donors were incubated at 37°C either for 5 days (RT-PCR) in cRPMI or for 12 days (ELISA and ELISPOT) in insulin and transferrin supplemented cRPMI with IL-10-secreting Tr1 or $CD4^+CD25^{+/-}$ cells. After optimization of cell number, 2.5 x 10^6 PBMC were cultured with 4'000 isolated IL-10-secreting, Der p 1-specific T cells or with 33'000 $CD4^+CD25^{+/-}$ T cells in 6-well-plates. 4'000 Der p 1-specific, IL-10-secreting cells or 11'000 $CD4^+CD25^{+/-}$ T cells were co-cultured with 200'000 purified B cells in 96-well-plates.

Determination of IgE, IgG4 and IgA by real-time PCR, ELISA and ELISPOT

Refer to Methods in the Supplementary material.

Statistical Analysis

Statistical analysis was performed using the paired Student's t-test and the Wilcoxon Matched Pairs Signed Ranks test.

Results

IL-10-secreting Tr1 cells suppress IgE and induce IgG4 production

Antibody synthesis and secretion in response to protein antigens are stimulated by CD40-mediated signals and B cell-activating cytokines expressed by T cells. Therefore, it was hypothesized that allergen-specific, IL-10-secreting Tr1 cells, which represent the dominant subset specific for common environmental allergens in healthy individuals, may also have an effect on Ig regulation. In the following experiments, we used IL-4 and sCD40L to induce IgE and IgG4, because IL-10-secreting Tr1 cells and IL-10 alone induced only very little IgG4 and no IgE (Fig. S1 in the Supplementary material). Der p 1-specific, IL-10-secreting, Tr1 cells are isolated from PBMC of healthy individuals and their role on IgE and IgG4 production is investigated. As shown in Fig. 5A, Tr1 cells suppressed germline ε transcript expression and simultaneously, increased productive γ4 mRNA expression in IL-4- and sCD40L-stimulated PBMC. Consistently, quantification of the secreted immunoglobulins also verified our findings. Tr1 cells decreased IgE- and enhanced IgG4-secretion (Fig. 5B). Similar results are found considering the frequency of IgE- and IgG4-secreting plasma cells. Tr1 cells decreased the number of IgE-secreting plasma cells, but enhanced the number of IgG4-secreting plasma cells (Fig. 5C). For comparison, Der p 1 or bee venom phospholipase A2-specific IL-4-secreting and IFN-γ-secreting T cells were used in the same type of experiments. IL-4-secreting T cells significantly increased IgG4 and IgE production, whereas IFN-γ-secreting T cells did not affect IgE and IgG4 production (Fig. S2 in the Supplementary material).

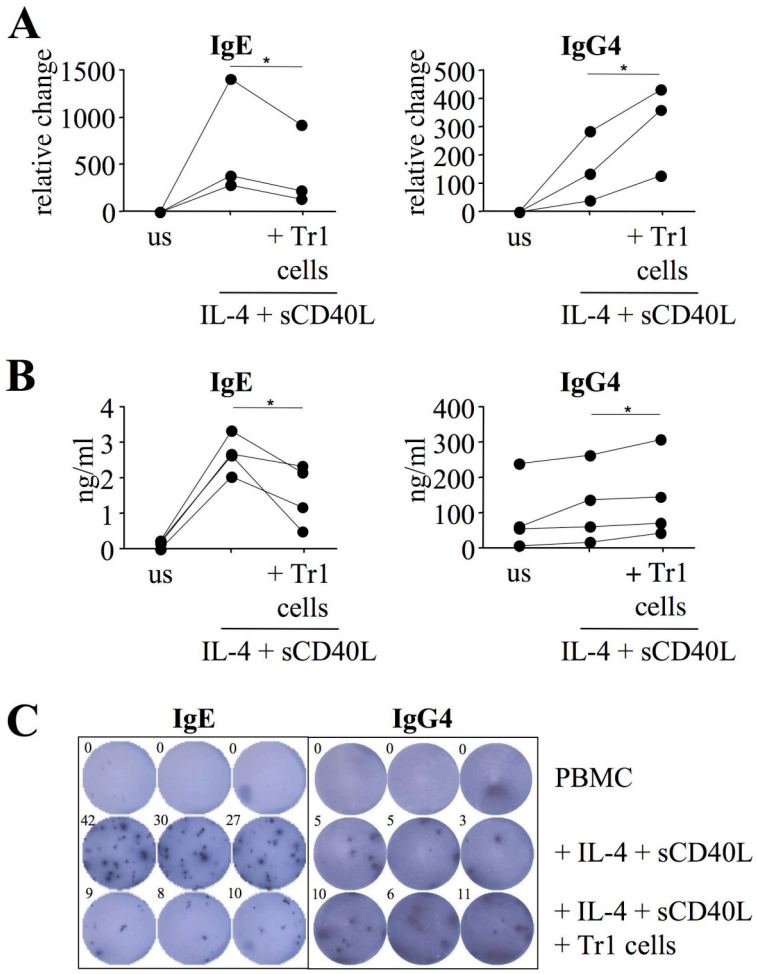

Figure 5. **Allergen-specific, IL-10-secreting Tr1 cells suppress IgE, but induce IgG4.** Der p 1-specific, IL-10-secreting Tr1 cells are isolated from PBMC from healthy donors and incubated together with PBMC. (A) Quantitative RT-PCR determined at day 5 (n=3). (B) Supernatants analyzed for IgE and IgG4 at day 12 (n=4). Conditions in the same experiment are linked with a line. (C) The frequency of IgE- and IgG4-secreting plasma cells measured at day 12 (1 representative of 3 independent experiments is shown). * $p < 0.05$.

Differential effect of $CD4^+CD25^+$ Treg cells on IgE and IgG4 production by PBMC from healthy individuals

We next investigated the influence of $CD4^+CD25^+$ Treg cells on immunoglobulin production. Similar to Tr1 cells, human peripheral blood $CD4^+CD25^+$ Treg cells also secrete IL-10 (222). Incubation of IL-4- and sCD40L-stimulated PBMC with $CD4^+CD25^+$ Treg cells from healthy individuals inhibited germline ε transcript expression, but they tend to induce productive γ4 transcript expression compared to $CD4^+CD25^-$ T cells (Fig. 6A). The same effects of $CD4^+CD25^+$ Treg cells were observed on the protein level. As shown in Fig. 6B, $CD4^+CD25^+$ Treg cells suppressed IgE secretion, whereas IgG4 production was enhanced by IL-4- and sCD40L-stimulated PBMC. Freshly purified $CD4^+CD25^+$ T cells did not induce IgE or IgG4 production in conditions where IL-4 and sCD40L were not used, suggesting that Th2 help is required for the production of these Ig isotypes (Fig. S1 in the Supplementary material). Not surprisingly, also the frequency of IgE- and IgG4-secreting plasma cells was decreased or enhanced, respectively, when $CD4^+CD25^+$ Treg cells were added to the IL-4- and sCD40L-stimulated PBMC cultures (Fig. 6C). $CD4^+CD25^-$ T cells did not influence IL-4 and sCD40L-stimulated IgE or IgG4 production.

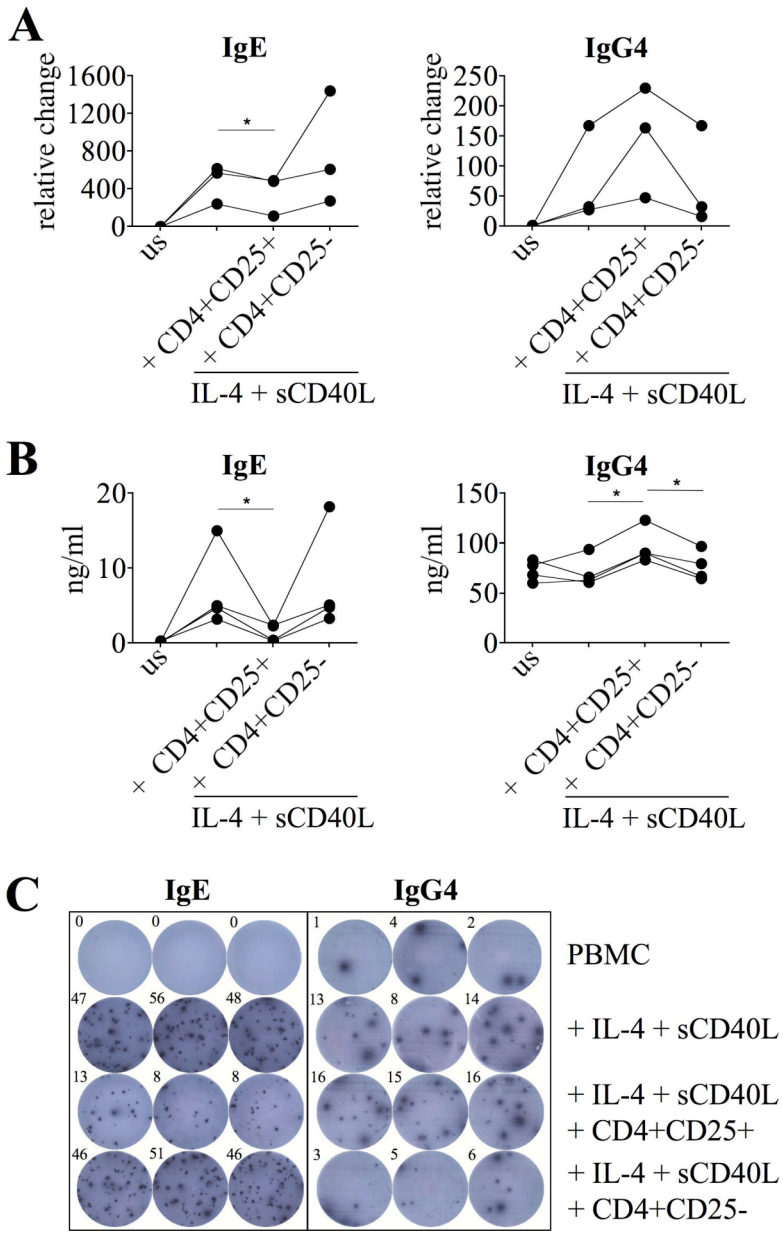

Figure 6. **Suppression of IgE and induction of IgG4 by $CD4^+CD25^+$ Treg cells.** $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells are isolated from PBMC from healthy donors and cultured together with PBMC. (A) Quantitative mRNA for RT-PCR measured at day 5 (n=3). (B) IgE and IgG4 concentrations in the supernatants at day 12 (n=4). (C) The number of IgE- and IgG4-secreting B cells analysed at day 12 (1 representative of 3 independent experiments is shown). * $p < 0.05$.

IL-10 counter regulates IgE and IgG4

On the basis of the results above and since IL-10 is one of the major cytokines of both Tr1 cells and $CD4^+CD25^+$ Treg cells, we next wanted to investigate the role of this cytokine on the Ig regulation. IL-10 decreased IL-4 and sCD40L-induced germline ε transcript expression (Fig. 7A). In contrast, IL-4 and sCD40L-induced productive $\gamma 4$ transcript expression is increased by IL-10. These effects of IL-10 could be observed in a dose-dependent manner in the range of 2 to 50 ng/ml. The findings in quantitative PCR are confirmed by determination of Igs in the supernatants of 12 day-cultures. PBMC stimulated with IL-4 and sCD40L showed decreased IgE secretion in the presence of IL-10 (Fig. 7B). In the same cultures, IL-10 synergized IL-4 and sCD40L-induced IgG4 secretion, whereas alone, it did induce only very small amounts of IgG4 (Fig. S1 in the Supplementary material). Similarly, IL-10 reduced the frequency of IgE-secreting plasma cells, whereas the IgG4-secreting plasma cell-frequency is enhanced (Fig. 7C). In unstimulated conditions, the frequency of IgE-producing plasma cells was practically negative. Only one spot per 50'000 cells was observed in 9 different experiments. The frequency of IgG4-producing plasma cells ranged between 0 to 4 spots per 50'000 resting PBMC. In IL-4- and sCD40L-stimulated cultures IgE spots increased to 27-59, IgG4 spots increased to 1-14 spots. Interestingly, the secreted protein of IgG4 was relatively high compared to IgE, suggesting utilization of IgE in cultures, such as binding to CD23 (309). Blocking of the IL-10 receptor in $CD4^+CD25^+$ Treg co-cultures demonstrated that their suppression effect on IgE and their induction effect on IgG4 are diminished (Fig. S3A in the Supplementary material). To strengthen the role of IL-10 as a soluble factor in the induction of IgG4, we performed transwell experiments, in which IL-10-secreting Tr1 cells were cultured in the upper well of the transwells and PBMC were cultured in the bottom of the transwells. IL-10-producing Tr1 cells induced IgG4 production without any requirement of cell-to-cell contact (Fig. S3B in the Supplementary material). In addition, IL-10 or IL-10Rα-chain were blocked by mAbs in the presence of IL-10. IgG4, which was significantly induced by IL-10 was almost 100% inhibited either by blocking the cytokine itself or its receptor (Fig. S3C in the Supplementary material).

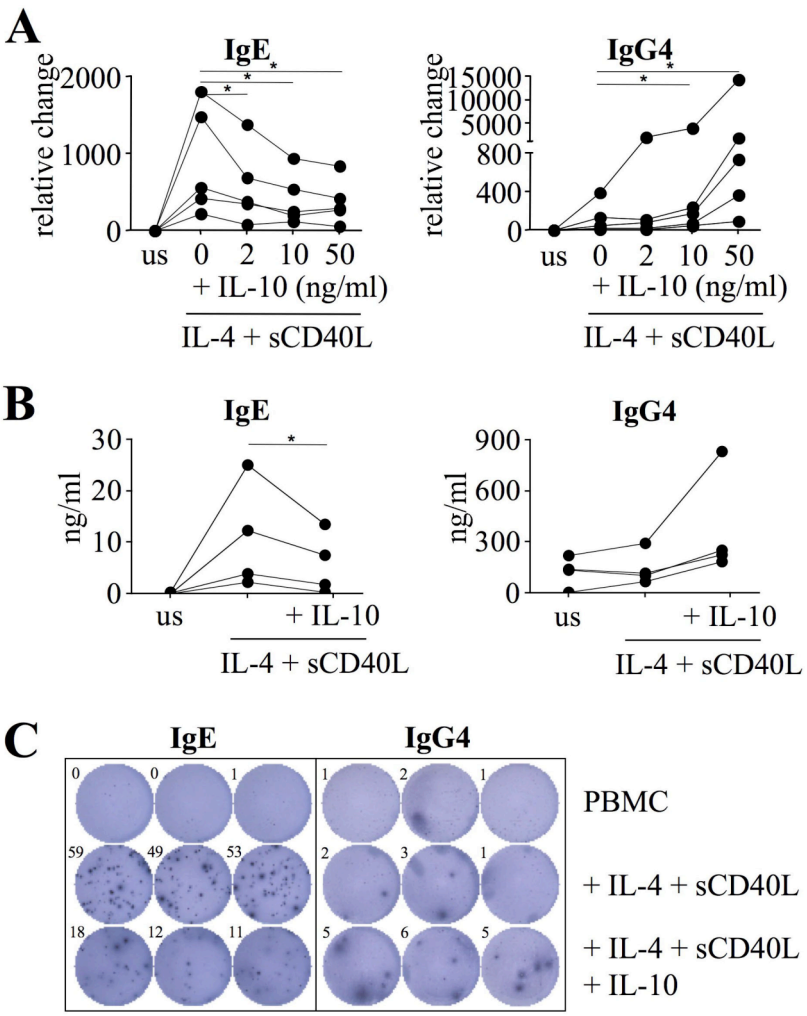

Figure 7. **IL-10 suppresses IgE and induces IgG4 production in PBMC.** Human PBMC from healthy donors are cultured with different concentrations of IL-10. (A) Quantitative RT-PCR measured after 5 days of incubation (n=5). (B) Immunoglobulin concentrations in the supernatants determined at day 12 (n=4). (C) The frequency of IgE- and IgG4-secreting plasma cells measured at day 12 (1 representative of 3 independent experiments is shown). (B), (C) IL-10 was used at 50 ng/ml. * $p < 0.05$.

Direct effect of Tr1 cells, $CD4^+CD25^+$ Treg cells and IL-10 on IgE and IgG4 production in pure B cells

To eliminate indirect effects of other cells in PBMC, we investigated the impact of IL-10-secreting Tr1 cells, $CD4^+CD25^+$ Treg cells and IL-10 on purified B cells. B cells were cultured in the presence of IL-4 and sCD40L. As observed in PBMC, addition of Der p 1-specific Tr1 cells, $CD4^+CD25^+$ Treg cells or IL-10 decreased germline ε transcript expression. Productive γ4 transcript expression was increased by Tr1 cells, $CD4^+CD25^+$ Treg cells and IL-10 (Fig. 8A). Considering the Ig-protein level, IgE secretion was diminished in the presence of Tr1 cells, $CD4^+CD25^+$ Treg cells or IL-10, but IgG4 secretion was strongly enhanced (Fig. 8B).

Therefore, we conclude, that antigen-specific, IL-10-secreting Tr1 cells and $CD4^+CD25^+$ Treg cells are able to reduce IgE production at the mRNA-, the protein- and the plasma cell frequency-level. Simultaneously, antigen-specific, IL-10-secreting Tr1 cells and $CD4^+CD25^+$ Treg cells can efficiently induce IgG4 production. The data is confirmed in 50 different experiments demonstrated in Fig. 5-8.

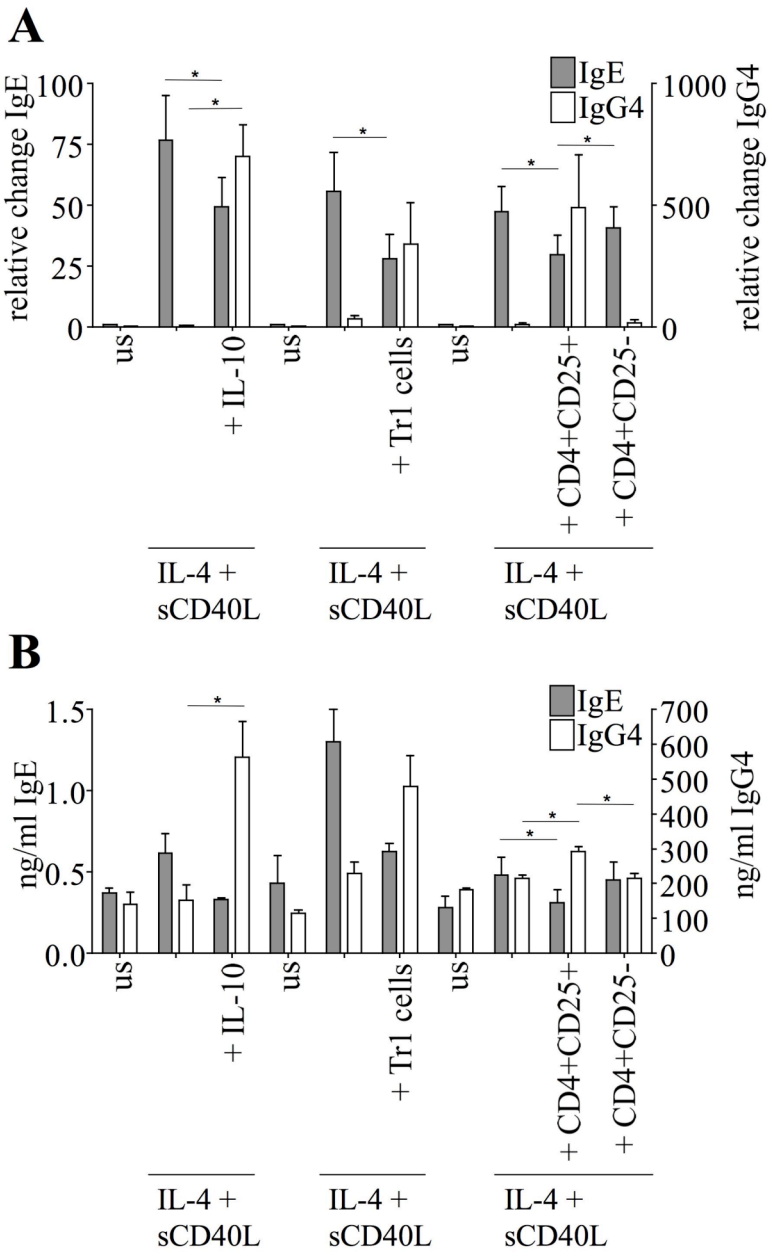

Figure 8. **IL-10-secreting Tr1 cells and $CD4^+CD25^+$ Treg cells regulate B cells.** B cells, IL-10-secreting Tr1 cells, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells are isolated from PBMC from healthy individuals. (A) Quantitative RT-PCR determined after 5 days of incubation of B cells with IL-10 (50 ng/ml), Tr1 cells, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells (mean values ± SE; n=9). (B) Immunoglobulin concentrations measured at day 12 in the supernatants (mean values ± SE; n=9). * $p < 0.05$.

IgA is induced by TLR7 or 9 stimulation but not by T cell help

Because neither Tr1 cells nor $CD4^+CD25^+$ Treg cells nor $CD4^+CD25^-$ T cells nor IL-10 showed any influence on IgA production in PBMC as well as in pure B cell cultures (Fig. S4A in the Supplementary material), we then investigated an array of TLR agonists for their capacity to induce IgA. PBMC were cultured in the presence of different TLR agonists. While stimulation of TLR2, 3, 4, 5, or 8 was not able to influence the IgA production (Fig. S4B in the Supplementary material), triggering of TLR7 or 9 highly enhanced both the mRNA level and the secretion of IgA compared to IgE and IgG4 (Fig. 9A and B). Interestingly, the addition of IL-4 and sCD40L to the cultures suppressed TLR7 or 9 induced IgA production, but upregulated IgE and IgG4 (Fig. S4B in the Supplementary material). Although dose dependent optimization was performed in preliminary experiments, TLR9 stimulation by type B CpG has always been the most potent stimulus compared to TLR7 for the induction of IgA. As a control, we also used type A CpGs, which could not induce IgA (Fig. S5 in the Supplementary material). We conclude that the IgA production is not influenced by Tr1 and $CD4^+CD25^+$ regulatory T cells, but rather induced by the innate immune system via activation of TLR7 or 9 stimulation.

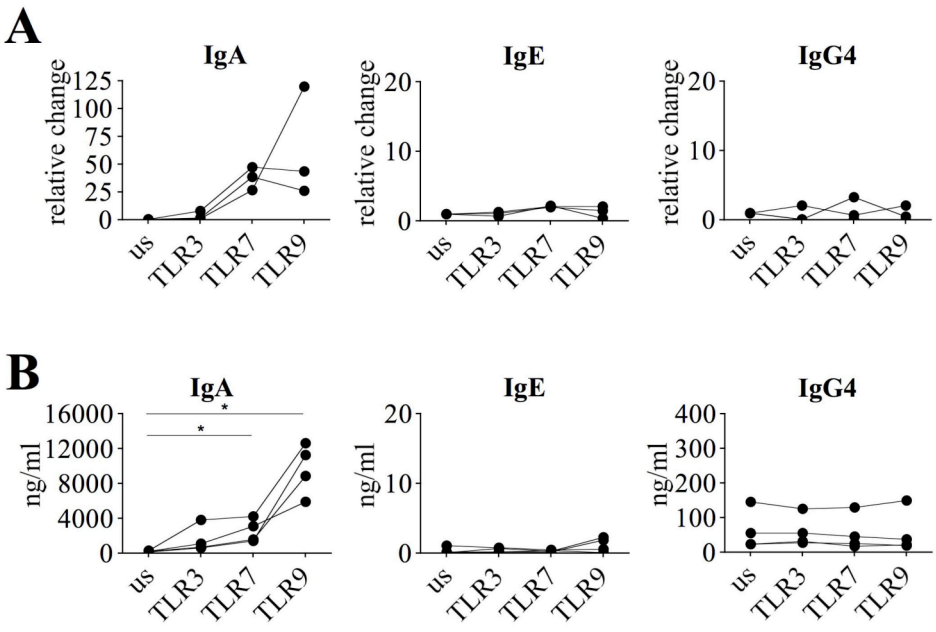

Figure 9. **Induction of IgA requires TLR7 and 9 triggering.** PBMC from healthy individuals are stimulated with TLR3, 7 or 9 ligands. (A) Quantitative RT-PCR determined after 5 days of incubation (n=3). (B) IgA, IgE and IgG4 concentrations in the supernatants measured after 12 days (n=4).

Discussion

In the present study, we demonstrate that the function of T regulatory cells is not only to suppress Th1 or Th2 cell responses, but that those cells also have direct influence on B cells. Tr1 cells and $CD4^+CD25^+$ Treg cells suppressed IgE and induced IgG4. Both regulatory T cell subsets also lowered IgE-secreting, and simultaneously augmented the IgG4-secreting plasma cell-frequency. In lymph nodes and tonsils, $Foxp3^+$ Treg cells are present in B cell areas where T-B cell interaction and humoral immune responses occur, and can directly suppress B cell Ig production and class switch recombination without a prerequisite to suppress Th cells. This is supported by studies, which show decreased Treg cell function or number in several immune mediated diseases in humans and mouse models with increased autoantibody production (310,311).

Studies on the mechanisms of immune responses to allergens have demonstrated that Treg cells are dominant in healthy individuals (87,312). If a detectable immune response is mounted, Tr1 cells specific for common environmental allergens, consistently represent the dominant subset. Healthy and allergic individuals exhibit the Th1, Th2 and Tr1 type, allergen-specific subsets in different proportions (87). Accordingly, a change in the dominant subset and the balance between Th2 and Treg cells may lead to either allergy development or recovery. $CD4^+CD25^+$ Treg cells have been associated with the spontaneous remission of Cow's milk allergy. Children who outgrew their allergy (tolerant children) had higher frequencies of circulating $CD4^+CD25^+$ T cells and decreased *in vitro* proliferative responses to bovine beta-lactoglobulin in PBMC compared with children who maintained clinically active allergy (313). Several studies have suggested that children with a cat at home have a decreased risk of sensitization and asthma. Children exposed to more than 20 µg of Fel d 1 per 1 g of house dust display an IgG4 antibody response to Fel d 1 without IgE antibody. This modified Th2 response is not associated with symptoms and has been regarded as a form of immunological tolerance (314).

Although peripheral T cell tolerance is rapidly induced during specific immunotherapy (SIT), there is no evidence for B cell tolerance in the early course (315). Especially IgG4, which increases during the course of SIT or natural high dose exposure, is thought to capture the allergen before reaching the effector cell-bound IgE, and thus to prevent the activation of mast cells and basophils (316). Allergen-specific IgG4 may be directed against the same epitopes as IgE, resulting in direct competition for allergen binding and a "blocking" effect (215). It has been proposed that successful specific immunotherapy is associated with an increase in

IgG blocking activity that is not solely dependent on the quantity of IgG antibodies (215,218).

The non-inflammatory role for IgG4 may be because the IgG4 hinge region has unique structural features that result in a lower affinity for certain Fcγ receptors and the ability to separate and pair again, leading to bi-specific antibodies that are functionally monomeric (44,45). Furthermore, IgG4 does not fix complement and is unable to form large immune complexes. In an allergen-specific immunotherapy study, in which well-defined recombinant allergen mixtures were used, all treated persons developed very strong allergen-specific IgG4 and also increased IgG1 antibody responses. Some patients, who were not initially sensitized to *Phleum pratense* group 5 (Phl p 5), developed strong specific IgG4, but not IgE antibody responses to that allergen (317).

IL-10 is a potent suppressor of both total and allergen-specific IgE, while it simultaneously increases IgG4 production (98,129). Thus, IL-10 not only generates tolerance in T cells; it also regulates specific isotype formation and skews the specific response from an IgE to an IgG4 dominated phenotype. The healthy immune response to Der p 1 demonstrated increased specific IgA and IgG4, small amounts of IgG1 and almost undetectable IgE antibodies in serum (222). House dust mite-SIT did not significantly change specific IgE levels after 70 days of treatment; however, a significant increase in specific IgA, IgG1 and IgG4 was observed (222). Most probably the decrease in IgE/IgG4 ratio during allergen-SIT is a feature of skewing an allergen-specific Th2 to a Treg cell predominance. However, although Treg cell generation happens within days, a significant decrease in IgE/IgG4 ratio occurs after several months. The reason for the long-time gap between the change in T cell subsets, but not IgE/IgG4 levels is not easily explainable by the half-life of antibodies. In this context, the role of bone marrow-residing IgE-producing plasma cells with very long lifespan remains to be investigated (318). To increase the efficacy of allergen-SIT a search for good adjuvants is efficiently being pursued. Usage of TLR7/9 triggering substances together with recombinant proteins may have a beneficial effect in allergen-specific immunotherapy. In this way, the blocking antibody isotype IgA is expected to increase simultaneously and support or even synergize the advantageous effects of IgG4 (218,319).

In this study, we demonstrate that IgA production is neither influenced by IL-10 nor by IL-10 secreting Tr1 cells nor by $CD4^+CD25^+$ Treg cells. This antibody isotype is particularly regulated by activation of the innate immune system. Addition of TLR7 or 9 agonists to PBMC cultures highly upregulated IgA production and secretion, whereas T cell-help to B cells seems not to be necessary, since Tr1 cells,

$CD4^+CD25^+$ Treg cells as well as IL-4 and sCD40L did not have any effect on IgA. Recent evidence indicates that TLR stimulation on B cells can control immunoglobulin isotype switch. TLR and MyD88 are required for class switching to the pathogenetic IgG2a and IgG2b isotypes, but not for the development of IgM autoantibodies (186). Furthermore, CpG, but not LPS, upregulates T-bet expression in B cells and induces IgG2a, whereas it decreases IgG1 and IgE production (187,188). In human B cells, TLR9 stimulation by CpG-DNA, in association with IL-10, initiates germline immunoglobulin heavy chain constant region Cγ1, Cγ2 and Cγ3 gene transcription (190). Besides having a direct effect on B cells, TLR agonists can affect B cell differentiation and isotype switch indirectly through activation of DCs and subsequent release of IFN-α and IL-6. Recently, it has been shown that gut DCs induce IgA class switch by synergistic effects of retinoic acid, IL-6 and IL-5 (195), however, the role of TLRs in DC-mediated IgA induction remains to be elucidated. In another study, IFN-α-, IFN-γ-, LPS- or CD40L-stimulated human dendritic cells up-regulated BAFF and APRIL, which induced together with IL-4, IL-10 or TGF-β CD40-independent class-switch recombination to Cγ, Cα or Cε in B cells (192). In addition, TLR ligands and APRIL were shown to synergize to further enhance IgA2 class switching (320). Moreover, it has been demonstrated that inducible-nitric-oxide-synthase (iNOS) induces TGF-βRII on B cells and controls T-cell-dependent IgA class-switch recombination. Furthermore, MALT DCs express iNOS in response to the recognition of commensal bacteria by TLRs, which in turn leads to the production of BAFF and APRIL and subsequent T-cell-independent IgA class-switch recombination in B1 cells (321).

If the mechanism by which regulatory T cells act on B cells consists of physical cell-to-cell contact via intercellular adhesion molecule 1 (ICAM-1), TGF-β (130), cytotoxic T cell-associated antigen-4 (CTLA-4) (322), glucocorticoid-induced tumor necrosis factor receptor (GITR) (323), programmed death-1 (PD-1) (324) and OX40 (CD134) (325), or, as very recently shown, via cyclic adenosine monophosphate and gap junctions (326), or if it is more an autocrine mechanism with IL-10 and TGF-β as soluble mediators, is not known yet. It appears however, that this antibody isotype regulation is partially dependent on IL-10 as demonstrated in our inhibition experiments with anti-IL-10 and anti-IL-10 receptor-antibodies and the transwell experiment (Fig. S3 in the Supplementary material).

We conclude that peripheral tolerance utilizes multiple mechanisms to suppress allergic inflammation. Apparently, Treg cells contribute to the control of allergen-specific immune responses in several ways: Suppression of antigen-

presenting cells that support the generation of effector Th2 and Th1 cells; suppression of Th2 and Th1 cells; suppression of mast cells, basophils and eosinophils; interaction with resident tissue cells and remodeling (88). In addition to the above mechanisms, the present study demonstrates suppression of IgE and induction of the non-inflammatory antibody isotype IgG4 by Tr1 and $CD4^+CD25^+$ Treg cells.

Acknowledgments

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Supplementary material

Methods

Determination of IgE, IgG4 and IgA

Quantitative real-time PCR: After 5 days in cell culture, cells were lysed with RNeasy lysis buffer, and the RNA was isolated using the RNeasy mini kit (QIAGEN, Hilden, Germany) and eluted in 50 µl ddH_2O. Reverse transcription was performed with RevertAid M-MuLV Reverse Transcriptase, Random Hexamer Primers, dNTP Mix, Ribo Lock RNase Inhibitor (all from Fermentas Burlington, Canada). The primers detecting germline transcripts of IgE (Iε-Cε), productive transcripts of IgG4 (J_H-Cγ4) and productive transcripts of IgA (Cα-Cα) were designed from appropriate database sequences using the PrimerExpress software (Applied Biosystems). Primers (all Microsynth, Balgach, Switzerland) used were as follows: IgE forward primer, 5'-ACA CAT CCA CAG GCA CCA AA-3', IgE reverse primer, 5'-TTG CAG CAG CGG GTC AA-3'; IgG4 forward primer, 5'-ACC C/ATG GTC ACC GTC TCC TCA-3', IgG4 reverse primer, 5'-GGG ACC ATA TTT GGA CTC-3'; IgA forward primer, 5'-CGC TGG CCT TCA CAC AGA A-3', IgA reverse primer, 5'-CGC CAT GAC AAC AGA CAC A-3'. cDNAs were amplified using SYBR®-PCR mastermix (Applied Biosystems, Branchburg, NJ, USA) according to the recommendations of the manufacturer in a total volume of 25 µl in a sequence detection system (ABI PRISM 7000; Applied Biosystems). Relative quantification was performed as described previously (87). The obtained values were normalized to the amount of 18S rRNA present in each sample. All amplifications were performed in triplicates.

ELISA: Secreted immunoglobulins were detected by ELISA on day 12. ELISA plates (Nunc, Roskilde, Denmark) were coated with 20 µg/ml purified mouse anti-human IgE (Novartis, Basel, Switzerland), 1 µg/ml purified mouse anti-human IgG4 or 1 µg/ml purified mouse anti-human IgA (both from BD Pharmingen, San Diego, CA, USA) Abs. IgE and IgG4 plates were blocked with PBS containing 1% BSA (Fluka, Buchs, Switzerland), IgA plates with PBS containing 2.5% milk powder. Supernatants were added to the plates and incubated at room temperature. Plates were then washed with PBS containing 0.05% Tween 20 (Fluka, Buchs, Switzerland) and incubated with biotinylated isotype-specific secondary antibodies (for IgE-ELISA from Novartis, Basel, Switzerland; for IgG- and IgA-ELISA from BD Pharmingen, San Diego, CA, USA) followed by extravidin peroxidase conjugate (Sigma-Aldrich, Steinheim, Germany). The assay was developed with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich, Steinheim, Germany) as chromogenic substrate. All measurements were performed in duplicates.

ELISPOT: The frequency of Ig-secreting cells was determined by ELISPOT on day 12. Filter plates (Pall Corporation, Ann Arbor, USA) were coated with 20 μg/ml purified mouse anti-human IgE (Novartis, Basel, Switzerland) or 4 μg/ml purified mouse anti-human IgG4, RJ4 (Beckman Coulter, Fullerton, CA, USA) Abs. The plates were blocked with PBS containing 1% BSA (Fluka, Buchs, Switzerland). 50'000 cells/well were added to the plates and incubated overnight at 37°C. Plates were then washed with PBS containing 0.05% Tween 20 (Fluka, Buchs, Switzerland) and incubated with a biotinylated IgE-specific secondary antibody (Novartis, Basel, Switzerland) followed by ExtrAvidin-Alkaline phosphatase (AP) conjugate (Sigma-Aldrich, Steinheim, Germany) or they were incubated with an anti-human IgG-AP conjugate. The assay was developed with 5-Brom-4-Chlor-3-Indolylphosphat/Nitroblau Tetrazolium (BCIP/NBT) (BIO-RAD, Reinach, Switzerland) as chromogenic substrate. All measurements were performed in triplicates.

CpG oligonucleotides, antibodies

Type B CpG2006 and the two type A CpGs, CpG2216 and CpG10, were all from Microsynth GmbH, Balgach, Switzerland. For sequences see reference (327). Anti-IL-10 mAb was purchased from R&D systems.

Transwell membrane system

We used transwell permeable supports from Corning Incorporated, NY, USA (3.0 μm polycarbonate membrane, 6.5 mm insert, 24 well plate). 6'600 IL-10-secreting Tr1 cells were cultured in the upper compartment of the transwells and $1.5x10^6$ PBMC were cultured in the lower compartment. Quantitative RT-PCR was performed after 5 days of incubation.

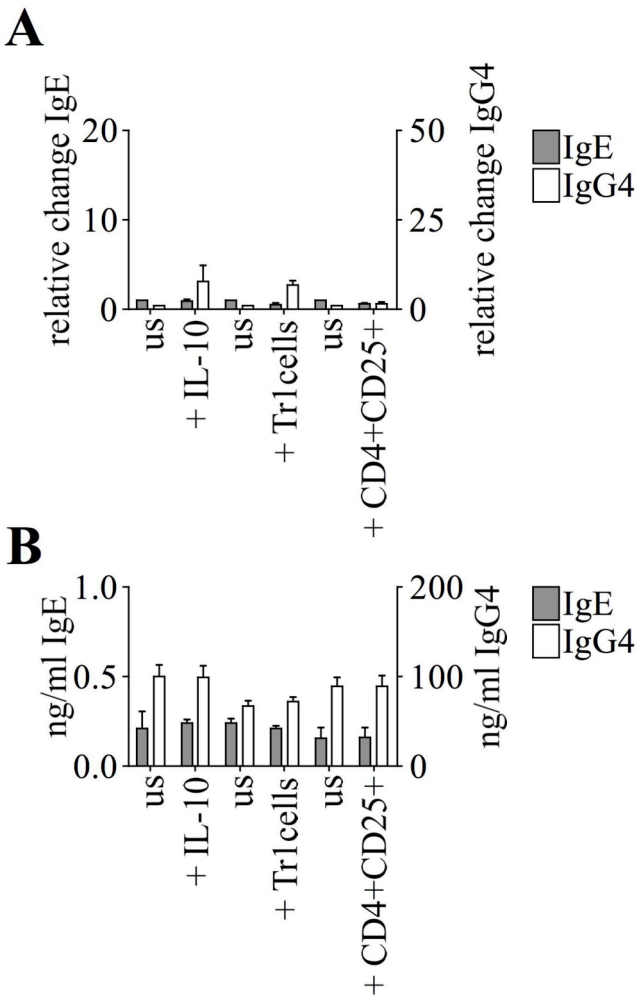

Figure S1. **IgG4 and IgE are not strongly induced by IL-10, IL-10-secreting Tr1 cells or $CD4^+CD25^+$ Treg cells without IL-4 and sCD40L.** PBMC from healthy individuals were incubated with IL-10 (50 ng/ml), IL-10-secreting Tr1 cells or $CD4^+CD25^+$ Treg cells. (A) Quantitative RT-PCR determined after 5 days of incubation (mean values ± SE; n=4). (B) The IgE and IgG4 concentrations in the supernatants measured after 12 days of incubation (mean values ± SE; n=4).

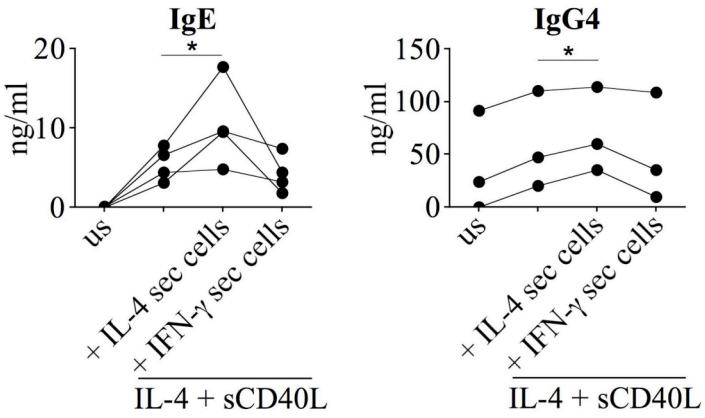

Figure S2. **Regulation of IgE and IgG4 by IL-4- and IFN-γ-secreting cells.** Der p 1 or bee venom phospholipase A2-specific IL-4-secreting and IFN-γ-secreting T cells were used as controls. IL-4-secreting T cells significantly increased IgG4 and IgE production, whereas IFN-γ-secreting T cells did not affect IgE and IgG4 production.

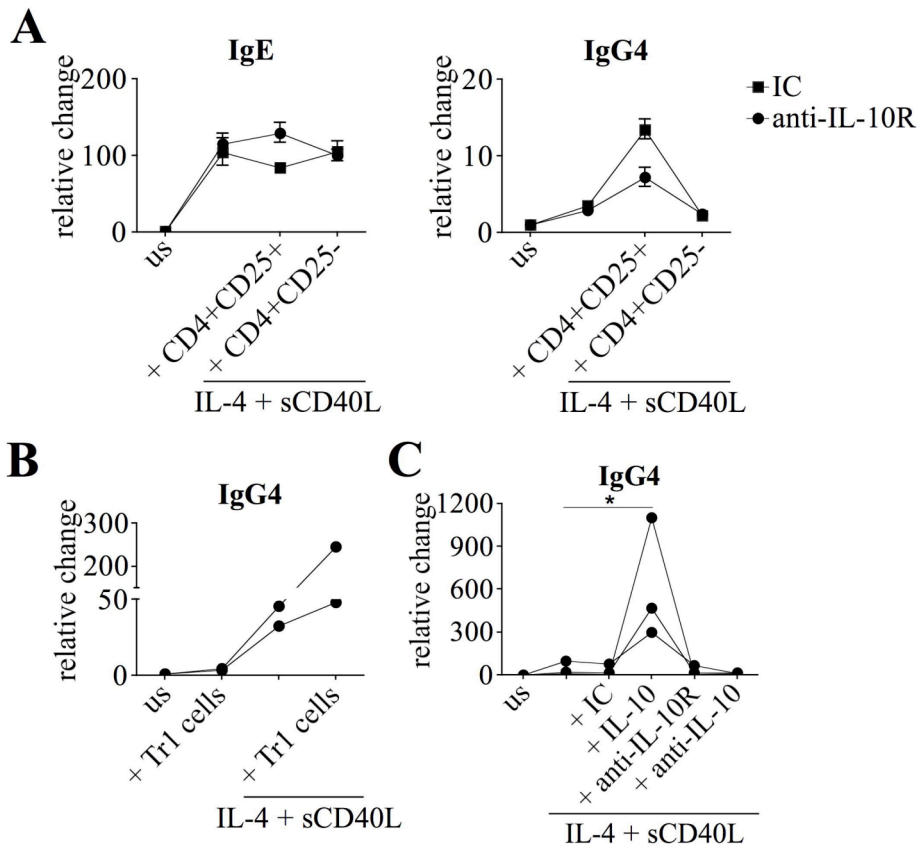

Figure S3. **Role of IL-10 in suppression of IgE and induction of IgG4 by Treg cells.** B cells from healthy individuals are incubated with $CD4^+CD25^+$ Treg cells or with $CD4^+CD25^-$ T cells. Quantitative RT-PCR determined after 5 days of incubation. (A) IgE germline transcripts and IgG4 productive transcripts (one representative experiment). (B) IL-10-secreting Tr1 cells were cultured in the upper compartment of the transwells and PBMC were cultured in the lower compartment. IL-10-producing Tr1 cells induced IgG4 production without any requirement of cell-to-cell contact (data represent 2 independent experiments). (C) In PBMC, IL-10 or IL-10Rα-chain was blocked by mAbs in the presence of IL-10. IgG4, which was significantly induced by IL-10 was strongly inhibited either by blocking the cytokine itself or its receptor (data represent 3 independent experiments) * $p < 0.05$. IC: isotype control.

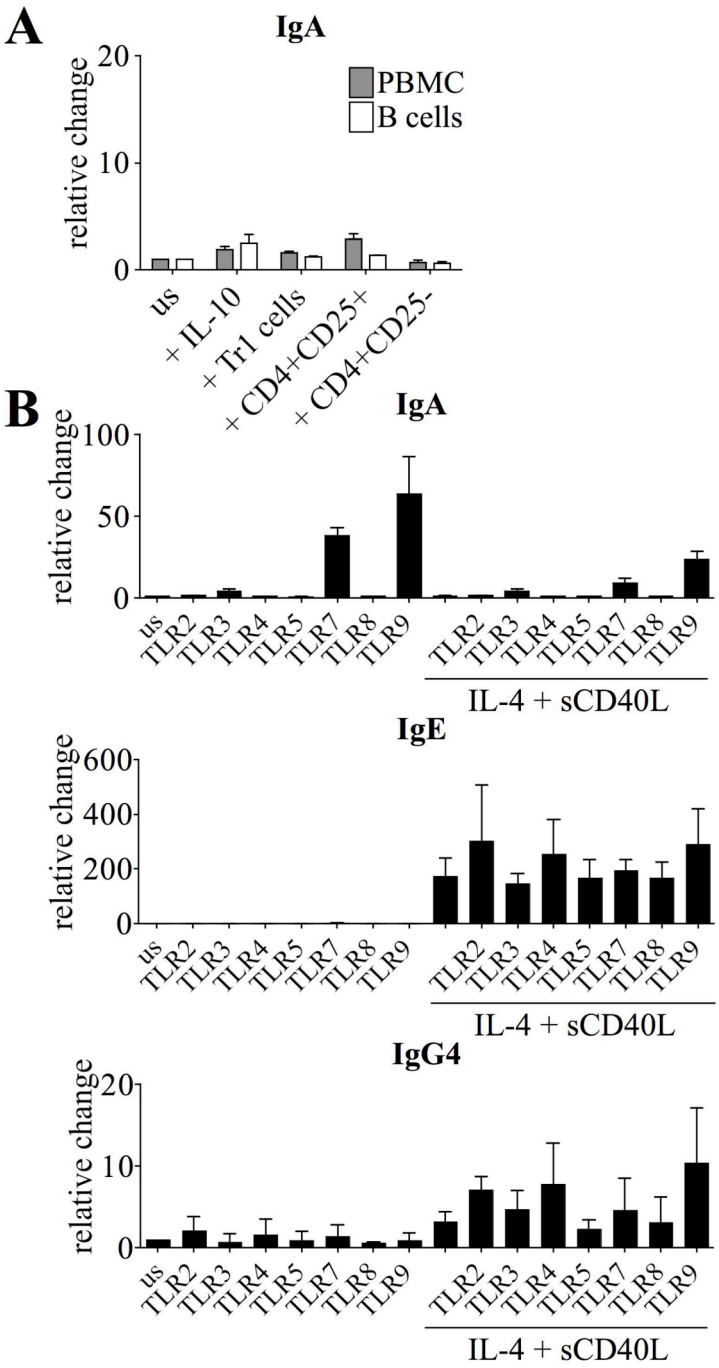

Figure S4. **IgA is elicited by stimulation of TLR7 or 9 but not by IL-10, IL-10-secreting Tr1 cells or $CD4^+CD25^+$ Treg cells.** (A) PBMC or pure B cells from healthy individuals are incubated with IL-10 (50 ng/ml), IL-10-secreting Tr1 cells or $CD4^+CD25^+$ Treg cells. Quantitative RT-PCR determined after 5 days of incubation (mean values ± SE; n=3-5). (B) PBMC from healthy individuals are stimulated with TLR2, 3, 4, 5, 7, 8 or 9 ligands. Quantitative RT-PCR determined after 5 days of incubation (n=3). IL-4 and sCD40L, which upregulated IgE and IgG4 suppressed TLR7 and TLR9 induced IgA.

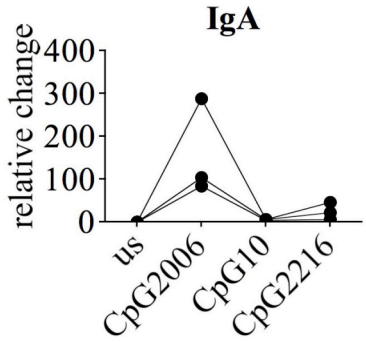

Figure S5. IgA was determined after stimulation of PBMC with type B CpG2006 and type A CpG10 and CpG2216 (data represent 3 independent experiments). CpG2006 was found to be the most potent inducer of IgA. In contrast, induction of IgA by A type CpGs was very weak. IgA mRNA was detected on day 5.

6.2 Clonal switch to IL-10-secreting type 1 T regulatory cells in high dose allergen exposure

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Running foot: T regulatory 1 cells in high dose allergen tolerance

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Key words: tolerance, T regulatory cells, allergy, histamine, immune regulation

Abbreviations: type 1 T regulatory: Tr1, cytotoxic T lymphocyte-associated antigen: CTLA, programmed death: PD, phospholipase A_2: PLA

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Abstract

High dose bee venom exposure in beekeepers by natural bee stings represents a model to understand mechanisms of T cell tolerance to allergens in healthy individuals. Continuous exposure of non-allergic beekeepers to high doses of bee venom antigens induces diminished T cell-related cutaneous late-phase swelling to bee stings in parallel with suppressed allergen-specific T cell proliferation and Th1 and Th2 cytokine secretion. After multiple bee stings, venom antigen-specific Th1 and Th2 cells show a clonal switch towards interleukin (IL)-10-secreting type 1 T regulatory (Tr1) cells. T cell regulation continues as long as antigen exposure persists and returns to initial levels within two to three months after bee stings. Histamine receptor 2 upregulated on specific Th2 cells displays a dual effect by directly suppressing allergen-stimulated T cells and increasing IL-10 production. In addition, cytotoxic T lymphocyte-associated antigen-4 and programmed death-1 play roles in allergen-specific T cell suppression. In contrast to its role in mucosal allergen tolerance, TGF-β does not seem to be an essential player in skin related allergen tolerance. Thus, rapid clonal switch to IL-10-producing Tr1 cells and the use of multiple suppressive factors represent essential mechanisms in immune tolerance to high dose of allergens.

Introduction

To avoid chronic cell activation and inflammation against nonpathogenic antigens through skin, ingestion and inhalation, the immune system has developed efficient peripheral tolerance mechanisms. Allergic diseases are due to an aberrant immune response mediated through a key effector cell, the T helper (Th) type 2 cell and associated cytokine pattern including IL-4, IL-5 and IL-13 (328). Consequently, the most pronounced findings with potential relevance to allergy therapy are related directly to the control of these Th2 immune effectors. There is strong evidence that peripheral T cell regulation plays a crucial role in the control of harmful T cell responses. Since the early 1970s many types of suppressor mechanisms have been demonstrated, and the biology of T regulatory (T_{reg}) cells has been the subject of intensive investigation (329-333).

Subsets of T_{reg} cells with distinct phenotypes and mechanisms of action include the naturally occurring, thymic selected $CD4^+CD25^+FoxP3^+$ T_{reg} cells, and the inducible type 1 T_{reg} cells (Tr1) (98,100,121,334-336). A great deal of uncertainty remains about differentiation factors, antigen specificity and mechanisms of action of T_{reg} cells. Several types of adaptive T_{reg} cells have been described with a unique mechanism of action that varies depending on the experimental model. T_{reg} cells act as suppressor T cells, which down regulate effector cells and antigen-presenting cells *in vitro* and in inflammation models such as various chronic infections, organ transplantation, allergy and autoimmunity (98,100,121,334-336). Although molecular mechanisms of T_{reg} cell generation remain to be elucidated, some existing therapies for allergic diseases such as treatment with glucocorticoids, and beta-2 agonists might function, to promote the numbers and function of IL-10-secreting Tr1-like cells (99,337).

Studies on the immune response to allergens provide well-defined models for understanding the regulation and circumvention of antigen-specific T-cell responses. The symptoms of immunoglobulin-E-mediated allergy–rhinitis, conjunctivitis and asthma can be ameliorated by the temporary suppression of mediators and immune cells (such as anti-histamines and corticosteroids) (92,338). However, the only long-term solution for the treatment of allergy is allergen-specific immunotherapy by the administration of high doses of allergen or allergen peptides that specifically target T cells over a long period of time (338). Successful venom and aeroallergen immunotherapy is associated with the induction of peripheral tolerance in T cells by generation of T_{reg} cells that secrete suppressive cytokines, IL-10 and TGF-β, suggesting that generation of Tr1 cells may play a role in healthy immune responses (87,312).

Investigation of allergen-specific peripheral T cell response in non-allergic beekeepers in and out of the bee keeping season has enabled us to study essential questions in peripheral T cell tolerance. One bee sting contains approximately 50 µg of protein consisting of several different antigens, which are directly inoculated into the skin. The major allergen phospholipase A_2 (PLA) is responsible for allergic reactions in almost all of the sensitized individuals and comprises approximately 10 to 20 % of bee venom's protein content (339). In the present study, we used several direct methods to analyse human specific T cell response in beekeepers, who were followed for consecutive years in and out of the bee keeping season. This represents a relevant model to investigate mechanisms of immune tolerance to high dose of allergen exposure in humans.

Results

High Dose Allergen Exposure Via Skin Induces Decreased Cutaneous Late-Phase Responses and Unresponsiveness in Allergen-Specific T Cells.

Beekeepers investigated in this study are not allergic to bee venom, but show significant cutaneous late-phase responses and peripheral blood T cell proliferation and cytokine production in response to bee venom major allergen, PLA. They do not use protective masks or gloves and receive numerous bee stings during their occupation. They start beekeeping in the last week of April and continue until October (Fig. 10A). Their exposure to high doses of venom antigens induces decreased cutaneous late-phase swelling response to bee stings within a few days, which continues throughout the whole season as long as antigen exposure persists. The size of skin reactions to bee stings return to initial levels a few months after the end of each season. In the beginning of the next beekeeping season, they experience the same degree of skin reactivity, again only in the first few bee stings (Fig. 10B). T cells play a major role in cutaneous late-phase responses and lesion size correlates with infiltrating T cell numbers (340). By monitoring peripheral blood T cell response in beekeepers for several years, a significant decrease in peripheral blood mononuclear cell (PBMC) proliferation against PLA, after multiple bee stings has been demonstrated (Fig. 10C). Approximately two months after the end of the beekeeping season, PLA-specific T cell proliferation returned to initial high levels. Beekeepers, who are followed two to three consecutive years showed the same T cell response profile every year, demonstrating a short lived peripheral T cell unresponsiveness, which depends on antigen exposure and its persistence. PBMC proliferative response to control antigens, PPD and tetanus toxoid did not show any change during bee stings (Fig. 10D).

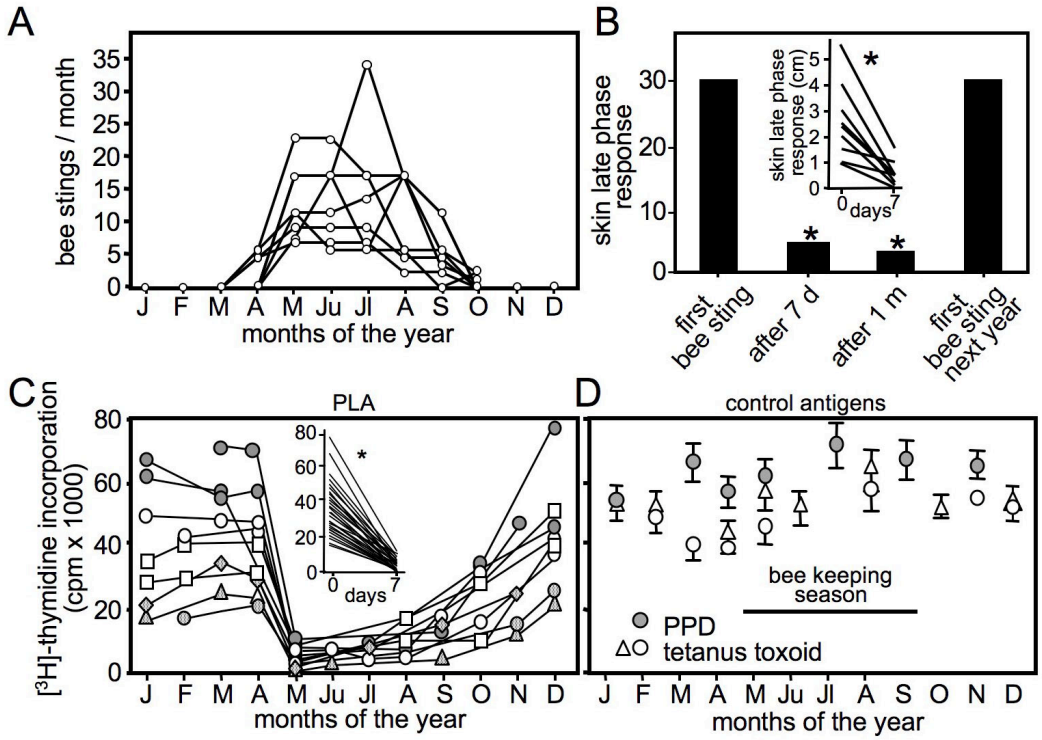

Figure 10. **Decreased antigen-specific T cell response and cutaneous late-phase response after natural high dose antigen exposure.** a) The number of beestings per month is demonstrated in 9 beekeepers b) Decreased cutaneous late-phase response after bee stings returns to initial levels upon no exposure (5 beekeepers followed for three years. Lesion size has been graded as 2: >3 cm, 1: 0-3 cm, 0:no swelling) and cutaneous late-phase response lesion size in 9 beekeepers (inner panel) in 2007 before and 7 days after bee stings c) PLA-induced [^{3}H] thymidine incorporation in PBMC after 5 days in two beekeepers followed for three and two beekeepers for two consecutive years (67 peripheral blood samples). PLA-induced [^{3}H] thymidine incorporations analysed in 29 experiments before and 7 d after bee stings between 2001 and 2007 (inner panel). d) T cell proliferation against control antigens (PPD and tetanus toxoid) did not show any change in 3 beekeepers throughout the year. (*:$p<0.001$).

Increased IL-10-Secreting Tr1-Like T cell Response Immediately After High Dose Venom Exposure.

To investigate the mechanism of T cell unresponsiveness, PLA-specific T cells were characterized before and after multiple bee stings. First, their frequency was determined according to their IL-4-, IFN-γ– and IL-10-secretion profile before and 7 days after bee stings. Although the overall frequency of PLA-specific T cells showed a slight increase from 12.4 to 14.8 in 10'000 $CD4^+$ T cells, a remarkable shift to IL-10-secretion in specific T cells was observed (Fig. 11A, B). In 10'000 $CD4^+$ T cells, the frequency of PLA-specific IL-4-secreting cells decreased from 1.23 ± 0.32 to 0.43 ± 0.17; IFN-γ-secreting T cells decreased from 5.66 ± 0.92 to 2.49 ± 0.76. In contrast, the frequency of PLA-specific IL-10-secreting T cells increased from 5.52 ± 1.93 to 11.87 ± 3.12. The shift in the frequency of cytokine-secreting T cells was strongly supported by changes in overall cytokine profile of PLA-specific T cells after multiple bee stings (Fig. 11C, D). Before bee stings and other times out of the bee keeping season (December to February), PLA-specific T cells showed an IL-10- and IFN-γ-predominant cytokine profile with relatively less IL-4 and IL-13 secretion. Again, 7 days after they received multiple bee stings, IL-10-production in PLA-stimulated T cells increased, whereas IFN-γ-, IL-4- and IL-13-production decreased.

As shown above, all three subsets of single allergen-specific T cells are present in healthy beekeepers and skew to IL-10-secreting T cells *in vivo*. Accordingly, their role on PLA-induced T cell proliferation and whether this is influenced by changing their ratios was investigated. We assayed the allergen-induced proliferation of IFN-γ-, IL-4- and IL-10-secreting T cells by adding those purified cells back into autologous PBMC. Their frequency was increased up to 10 times higher than initial levels in PBMC (Fig. 11E). PLA-, Bet v 1- and Der p 1-specific IL-10-secreting T cells were purified from the same beekeeper. PLA-induced T cell proliferation, which was abolished after bee stings was reconstituted by increasing the numbers of PLA-specific IFN-γ- and IL-4-, but not IL-10-secreting T cells. PLA-specific IL-10-secreting T cells suppressed PLA-stimulated proliferating T cells, which secrete IL-4 and IFN-γ. For comparison, Bet v 1- and Der p 1-specific IL-10-secreting T cells did not suppress PLA-stimulated T cell proliferation, demonstrating the allergen specificity of bee venom induced T cell unresponsiveness.

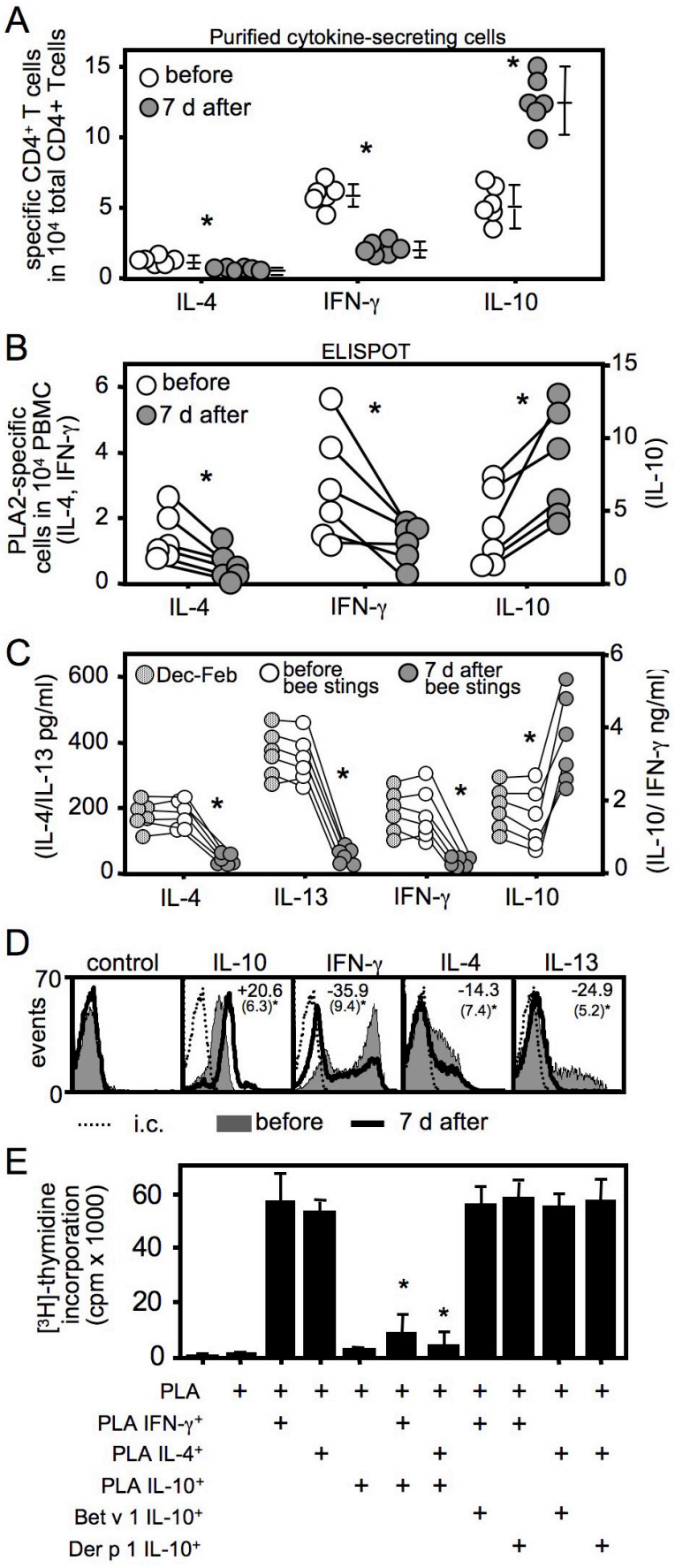
A
Purified cytokine-secreting cells
specific CD4+ T cells in 10⁴ total CD4+ Tcells
before
7 d after
IL-4
IFN-γ
IL-10
B
ELISPOT
PLA2-specific cells in 10⁴ PBMC (IL-4, IFN-γ)
(IL-10)
before
7 d after
IL-4
IFN-γ
IL-10
C
(IL-4/IL-13 pg/ml)
(IL-10/ IFN-γ ng/ml)
Dec-Feb
before bee stings
7 d after bee stings
IL-4
IL-13
IFN-γ
IL-10
D
control
IL-10
IFN-γ
IL-4
IL-13
events
+20.6 (6.3)*
-35.9 (9.4)*
-14.3 (7.4)*
-24.9 (5.2)*
i.c.
before
7 d after
E
[³H]-thymidine incorporation (cpm x 1000)
PLA
PLA IFN-γ+
PLA IL-4+
PLA IL-10+
Bet v 1 IL-10+
Der p 1 IL-10+

Figure 11. **Increased allergen-specific Tr1 and decreased Th1 and Th2 cells after bee stings.** a) PLA-specific IL-4-, IL-10- and IFN-γ-secreting $CD4^+$ T cells are purified and their frequency calculated in 6 beekeepers before the beginning of bee keeping season and after 7 d. They received an average of 13 bee stings. b) Frequency of PLA-stimulated cytokine-secreting cells in 6 beekeepers measured by ELISPOT assay. c) Secreted cytokines are measured in PLA-stimulated PBMC cultures (IL-4 after 24 h, IL-13, IFN-γ and IL-10 day 5) by ELISA. d) PBMC from beekeepers are stimulated with PLA for 7 d and intracytoplasmic cytokines were determined 12 h after anti-CD2/CD3/CD28 mAb stimulation. Mean percent difference (standard deviations are in brackets) of 8 experiments is shown before and 7 days after multiple bee stings inside each histogram. e) PLA- specific IL-4-, IL-10- and IFN-γ-secreting and Der p 1- and Bet v 1-specific IL-10-secreting T cells were purified from beekeepers after multiple bee stings. Their frequency was calculated in $CD4^+$ T cells and $2x10^5$ PBMC were immediately reconstituted by increasing their frequency in cultures by 10 times (IL-4-secreting T cells 0.02 % to 0.2 %; IL-10-secreting T cells 0.1 % to 1 %, IFN-γ-secreting T cells 0.05% to 0.5%). Cells were stimulated with PLA and [^{3}H]thymidine incorporation was determined after 5 d (similar results were obtained in three experiments). *:$p < 0.001$.

In Vivo Clonal Switch of Allergen-Specific Th2 and Th1 Cells Towards IL-10-Secreting Tr1 Cells.

After the observation of a change in T cell cytokine profile within 7 days *in vivo*, we investigated whether the increase in allergen-specific Tr1 cells occurs as a switch in cytokine profile and clonal expansion from specific memory Th1 and Th2 cells or new Tr1 cells are generated from the naïve T cell pool (Fig. 12). By using primers spanning different parts of T cell receptor (TcR) variable β chain, we demonstrated that purified PLA-specific IFN-γ-, IL-4- and IL-10-secreting T cells consisted of clonal T cells *in vivo*. After bee stings, their clonality increased particularly in IL-10-secreting T cells and decreased or disappeared in IFN-γ-secreting T cells. Very interestingly, the clonality of IFN-γ-secreting T cells disappeared and clonality in IL-10-secreting T cells appeared in a certain TcRvβ group of T cells detected by primer set D.

To further support the data on *in vivo* clonal switch to Tr1 cells, again PLA-specific IL-4-, IL-10- and IFN-γ-secreting T cells were purified and expanded with IL-2. $CD4^+$ T cells were stained with a panel of T cell receptor vβ chain mAbs and their frequency was determined (Fig. 13). 7 days after multiple bee stings, a switch in TcR vβ2 positive T cells from IL-4-secretion to IL-10-secretion profile was observed. In addition, TcR vβ12-bearing T cells skewed from an IFN-γ- and IL-4-dominant profile towards an IL-10-dominant profile. In another beekeeper, an increase in TcR vβ8-positive IL-10-secreting T cells and decreased TcR vβ17-positive IL-4- and IFN-γ-secreting T cells was demonstrated after bee stings. It has to be noted that the allergen-specific T cell population analysed here represent 0.1 to 0.01 % of all T cells. Together, these data demonstrate that an *in vivo* clonal switch occurs in allergen-specific $CD4^+$ T cells towards IL-10-secreting T cells after multiple bee stings.

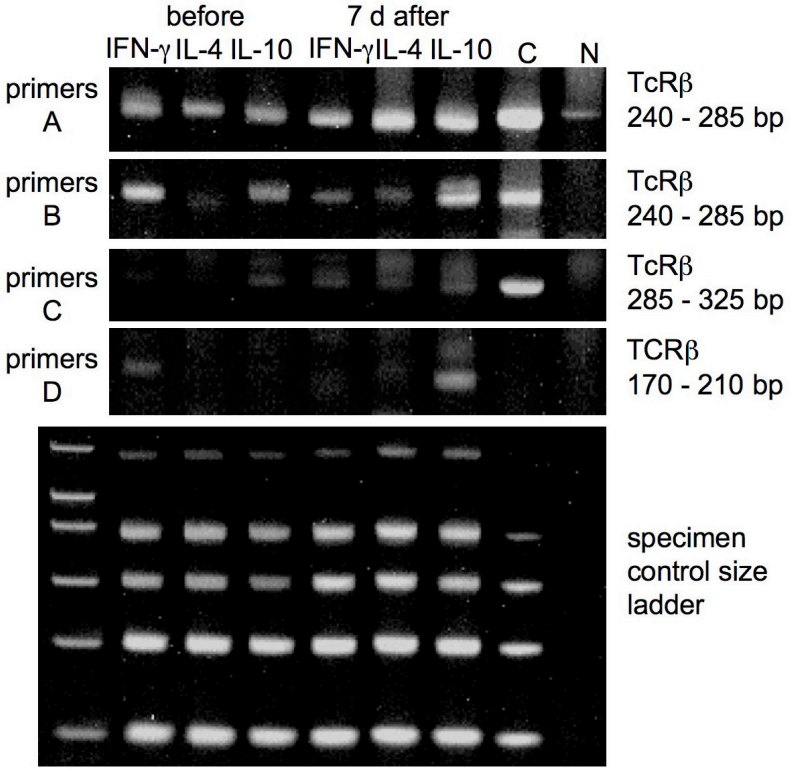

Figure 12. **Clonal skew to IL-10-secreting T cells after bee stings.** T cell receptor beta gene clonality is analysed in PLA-specific IL-4-, IL-10- and IFN-γ-secreting T cells before and 7 d after multiple bee stings. A clonal sample (C) and a non-clonal sample (N) are included as controls. The PCR products of three different test tubes are run on three different gels and a band which results from a clonal sample appears in a range from 240 bp – 285 bp (primers A, tube 1), 240 bp – 285 bp (primers B, tube 2), 170 bp – 210 bp and 285 bp – 325 bp (primers C and D, tube 3). The specimen control size ladder master mix generates a series of amplicons to ensure that the quality and quantity of input DNA is sufficient for the test. Heteroduplex analysis of the PCR products (except for the Specimen Control Size Ladder) on 6% TBE polyacrylamid gels, stained with ethidium bromide is shown. One representative from three experiments is shown.

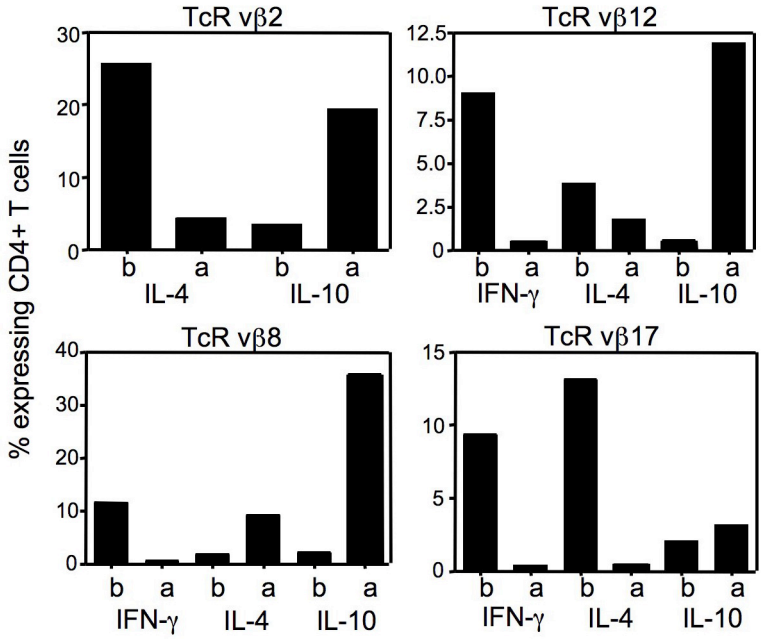

Figure 13. **In vivo switch from PLA-specific IL-4- and IFN-γ-secreting towards IL-10-secreting CD4$^+$ T cells in TcRVβ2-, 8- and 12- expressing T cells.** Two beekeepers were analyzed for TcRVβ chain expression in purified PLA-specific IL-4-, IL-10- and IFN-γ-secreting T cells by gating CD4$^+$ T cells by flow cytometry. The whole panel of TcR mAbs have been used for staining. Significant switch in certain TcR positive cell percentage between T cell subsets after bee stings is shown.

Role of Histamine Receptor 2 in Peripheral T Cell Tolerance.

Piecemeal degranulation of mast cells and basophils below the threshold of systemic anaphylaxis occurs by bee stings and histamine is released as one of the main mediators (339). Accordingly, we investigated the regulation of histamine receptors (HR) on allergen-specific T cells. HR2 showed a significant increase in allergen-stimulated T cells 7 d after multiple beestings (Fig. 14A). There was no change in HR1, HR4 and FoxP3 expression and PHA- and PPD-stimulated T cells. We further investigated the *in vivo* expression levels of HR2 mRNA in allergen-specific IL-4-, IFN-γ– and IL-10-secreting T cells immediately after purification (Fig. 14B). After bee stings, HR2 was exclusively upregulated on Th2-like IL-4-secreting T cells without showing any difference in IFN-γ- and IL-10-secreting T cells. The expression of FoxP3 was relatively low in allergen-stimulated cells compared to $CD4^+CD25^+$ cells purified from beekeepers and did not show any change after bee stings in both allergen-stimulated cultures and whole $CD4^+CD25^+$ population. There was also no change in $CD4^+CD25^+$ T cell percentages after bee stings (data not shown). Whether increased HR2 on Th2 cells has a role in IL-10 secretion in T cells was next investigated. PLA-specific IL-4-secreting T cells were stimulated with different doses of histamine in the presence or absence of HR2 antagonist ranitidine. Histamine dose dependently decreased IL-4 and IL-13 production and upregulated IL-10 production in T cells. This effect was specifically due to HR2 triggering, because of complete suppression by ranitidine (Fig. 14C).

The role of HR2 and IL-10 in allergen-specific T cell suppression was investigated by using an IL-10 receptor blocking mAb and ranitidine in PLA-stimulated T cells before and after bee stings. Before bee stings 3.22 ± 0.71 of $CD3^+CD4^+$ T cell showed divisions by PLA stimulation. IL-10 significantly suppressed this proliferation to background levels. 7 d after bee stings, PLA-specific proliferating $CD3^+CD4^+$ T cells significantly decreased to 0.86 ± 0.41. It was observed that most of the suppression induced 7 days after multiple bee stings has been recovered by blocking of IL-10 receptor and histamine receptor 2 and there was an additive effect of both as demonstrated in [^{3}H]-thymidine incorporation (Fig. 15A, B).

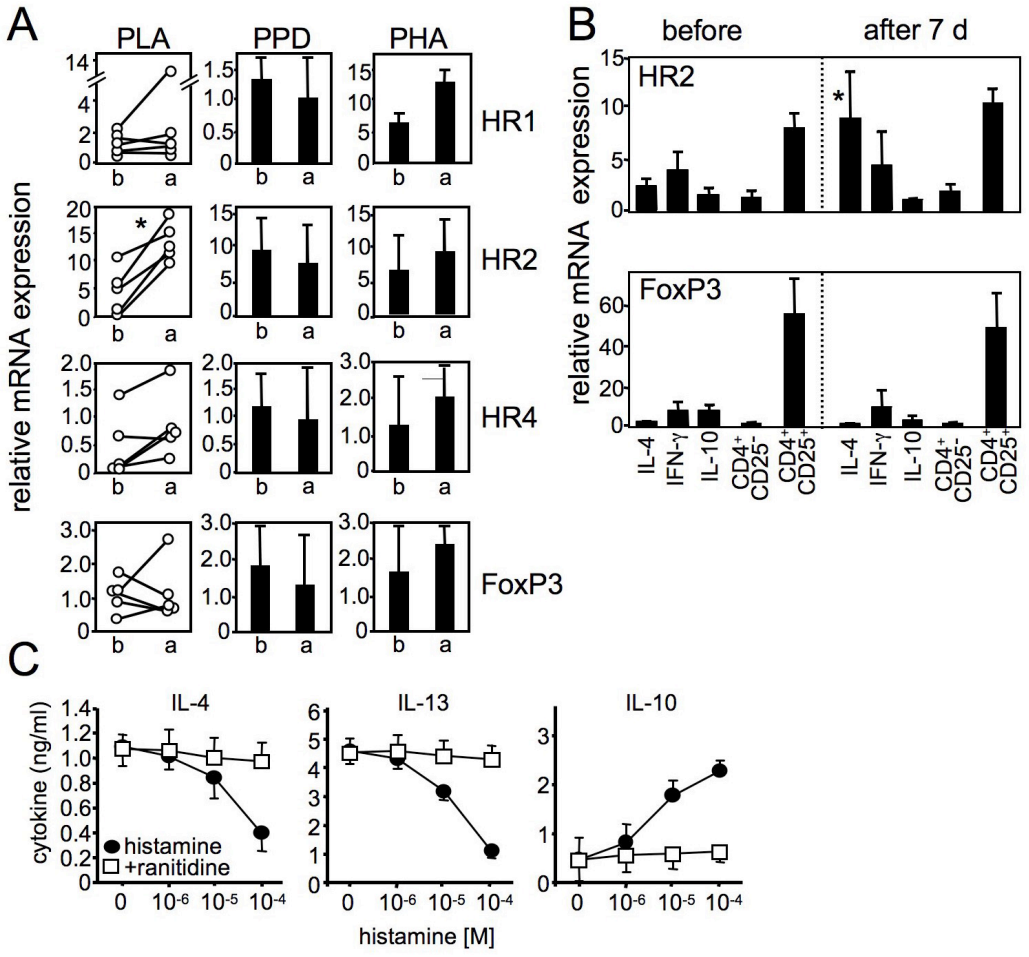

Figure 14. **HR2 is upregulated in specific T cells after multiple beestings and induces IL-10 production.** a) PBMC are stimulated with PLA for 7 days. HR1, HR2, HR4 and FoxP3 mRNAs were determined 4 hours after anti-CD3/ anti-CD28 mAb stimulation before and 7 d after multiple bee stings. For comparison PPD- and PHA-stimulated cells did not show any difference. b) HR2 and FoxP3 have been analysed in purified allergen specific IL-4-, IL-10- and IFN-γ-secreting T cells immediately after purification and $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. c) PLA-specific IL-4-secreting T cells were purified and stimulated with different doses of histamine in the presence or absence of 10^{-4} M ranitidine. Cytokines were determined by ELISA after 3 days.

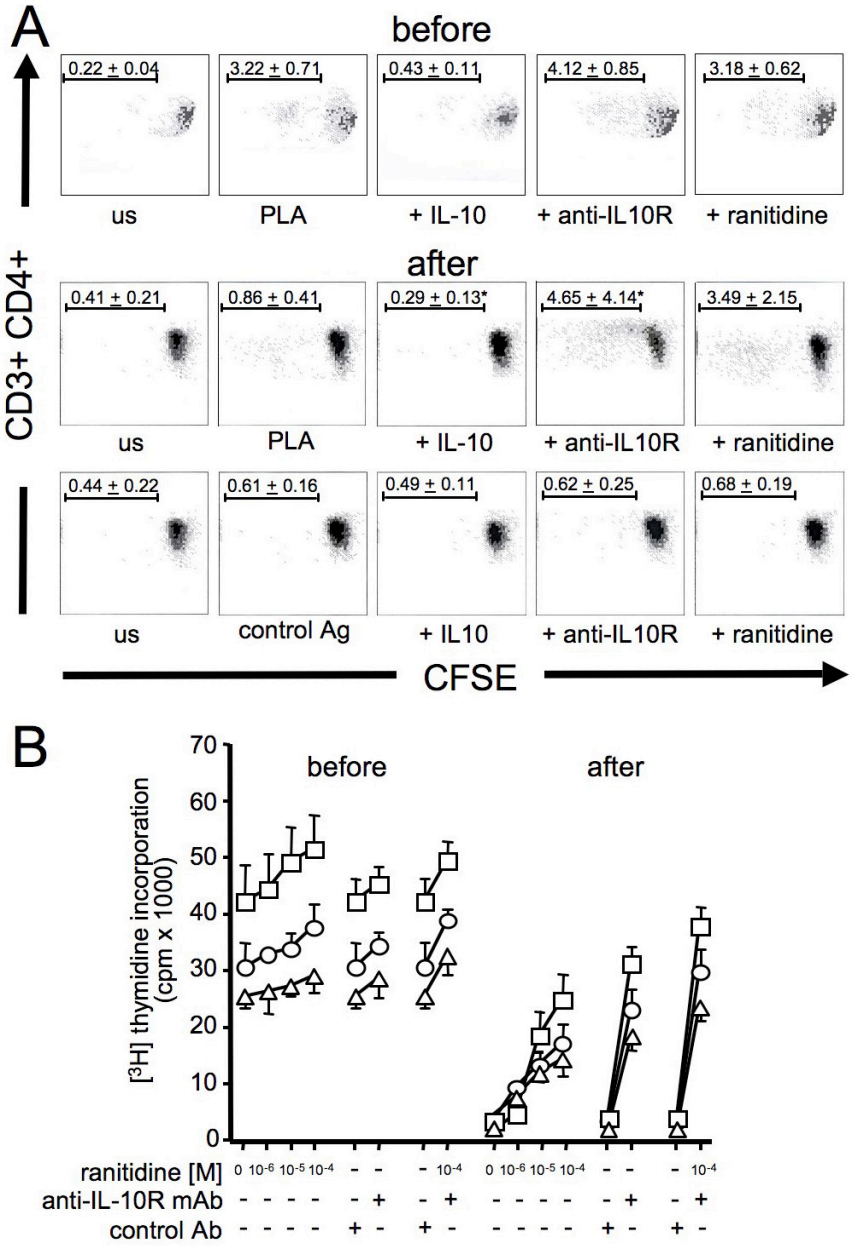

Figure 15. **HR2 and IL-10 play major roles in peripheral T cell tolerance to high dose antigen exposure.** a) PBMC of beekeepers were labeled with CFSE and dilutions in CFSE-expressing cells were analysed in the presence of IL-10, anti-IL10 receptor mAb (blocking) and 10^{-4} M ranitidine before and 7 days after multiple bee stings. Bovine serum albumine, tetanus toxoid and bee venom allergen hyaluronidase were used as control Ags in non responsive donors. On day 6, cells were collected, stained with anti-CD4 PE, and analyzed by FACS (one representative of 3 beekeepers is shown). b) PBMC of three different beekeepers were stimulated with PLA and different doses of ranitidine or anti-IL-10 receptor mAb or both. [^{3}H]-thymidine incorporation was determined at day 6.

Predominant Role of IL-10, but not TGF-β in Skin Related Tolerance.

Neutralization experiments revealed that multiple suppressive mechanisms play a role in suppression of allergen-specific Th2 cells. IL-10 and TGF-β has been suggested to co-operate in suppression of mucosal allergen-specific T cell activation against mucosal antigens (87). It appeared that IL-10 plays a more dominant role than TGF-β in suppression of bee sting and skin related PLA-specific T cell responses (Fig. 16A). Blocking of IL-10, but not TGF-β binding to its receptor, reconstituted the suppressed specific T cell proliferation and cytokine production after bee stings. The suppressive effect of IL-10 was equal in both Th1 cytokine IFN-γ and Th2 cytokine IL-13. In addition, two other mechanisms apparently function in immune tolerance to high dose venom allergens, because neutralization of either cytotoxic T lyphocyte-associated antigen (CTLA)-4 or programmed death (PD)-1 significantly blocked the Tr1 cell-mediated suppression of T cell proliferation (Fig. 16B). PLA-induced T cell proliferation was significantly high in PBMC of bee venom allergic donors. A suppression of T cell proliferation was achieved by increasing the frequency of PLA-specific IL-10-secreting T cells, which was partially inhibited by blocking of CTLA-4 or PD-1.

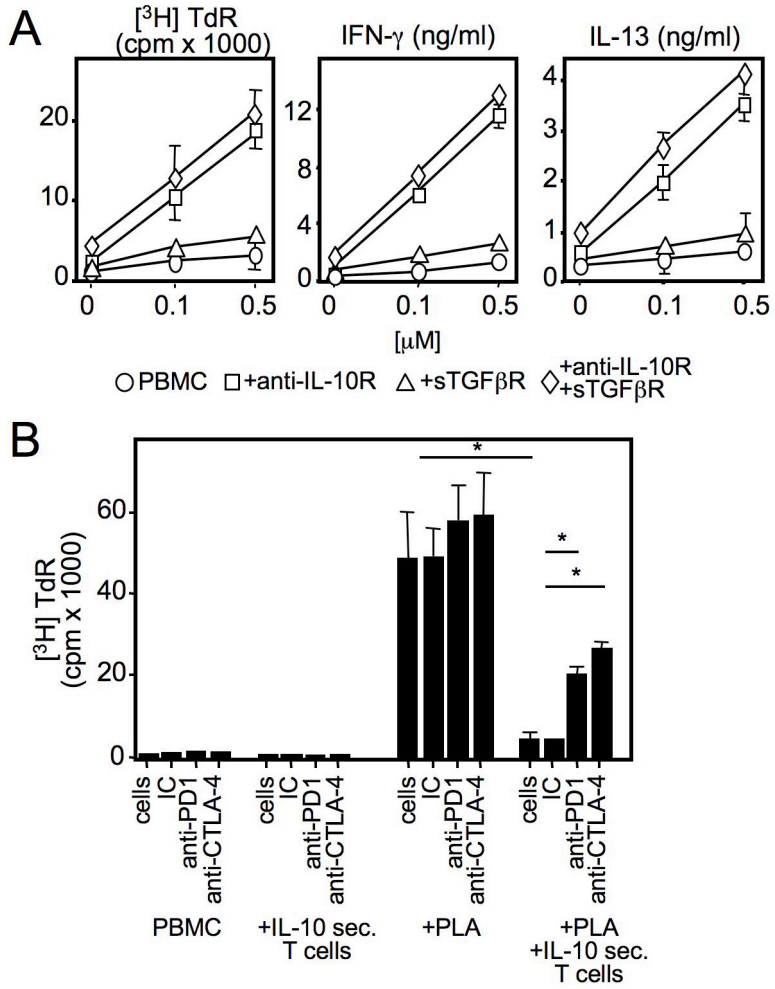

Figure 16. **Multiple suppressive mechanisms play a role in peripheral allergen tolerance.** a) Endogenous IL-10, TGF-β or both were neutralized in PLA-stimulated PBMC of beekeepers during bee keeping season. [^{3}H]-thymidine incorporation (TdR), IFN-γ and IL-13 were determined at day 5. b) PLA-specific proliferation of PBMC from beekeepers before multiple bee stings is suppressed by 10 times increased frequency of PLA-specific IL-10-secreting T cells. The activity of CTLA-4 and PD-1 were neutralized. [^{3}H]-thymidine incorperation was determined at day 6 (cells: no neutralizing Ab or isotype control antibody, IC: isotype control antibody). (a, b) Same results were obtained in six other independent experiments all performed with freshly purified cells without *in vitro* expansion (*: p<0.001).

Discussion

The immune system must distinguish between innocuous and pathological antigens to prevent unnecessary and self-destructive immune responses (341). The human model we envisaged demonstrates how soluble protein antigens encountered via skin exposure are tolerated. Compared to aeroallergen exposure, the bee venom model is better controlled and represents a much higher dose of allergen inoculation via skin. Beekeepers received an average of 13 beestings in one week and they did not develop fever, draining lymph node swelling or splenomegaly. Induction of clinical tolerance parallel to immunological tolerance should be emphasized here, since these beekeepers showed local cutaneous late-phase reactions characterized by local swelling during the first few bee stings of every new season, which significantly diminished after one week.

One of the key findings of the present study concerns the life span and allergen-dependence of allergen-specific peripheral T cell tolerance. The cytokine profile of memory T cells specific for PLA show an IL-10- and IFN-γ-predominant profile out of the bee keeping season. Upon bee venom exposure, an immediate switch of allergen-specific T cells from Th1 and Th2 cells towards Tr1 cells occurs within a few days. These sequential events are repeated every year. At the end of every season when there is no exposure, peripheral T cell response returns to the same levels before exposure within 2 to 3 months. The changes in cytokine profile, T cell proliferation and cutaneous late-phase response show parallel changes, demonstrating that this T cell tolerance induced by an in vivo switch to Tr1-like IL-10-secreting cells is short-lived and dependent on antigen exposure. There is so far no report on *in vivo* turnover and lifespan of Tr1 cells in humans, but our data is in agreement with the *in vivo* doubling time of $FoxP3^{+}CD25^{hi}CD4^{+}$ T cells, which was demonstrated to be relatively short (8 days) in resting conditions compared to $FoxP3^{-}CD25^{-}CD4^{+}$ T cells (24 days) (342). Interestingly, when bee venom contact ceased, the Tr1 response substantially decreased within maximum 2 months.

Another essential data of the present study demonstrates *in vivo* clonal switch in allergen-specific IL-4-producing and IFN-γ-producing T cells towards IL-10-producing T cells. The consistent profile of PLA-specific T cell proliferation in beekeepers throughout consecutive years demonstrates a biphasic T cell response with substantial peripheral T cell tolerance upon high dose allergen exposure. The long term PLA-specific T cell repertoire has been maintained by average balance of 42% IFN-γ-secreting T cells, 43% IL-10-secreting T cells and 15 % IL-4 secreting T cells. Immediately, after bee stings the repertoire changed and consisted of 80% IL-

10-secreting T cells, 14% IFN-γ-secreting T cells and 6% IL-4-secreting T cells. The stability of cytokine profiles in differentiated effector and memory T cell subsets in humans is not fully known and it has been demonstrated that lineage committed memory T cell subsets are responsive to cytokine signals of the opposing lineage (343,344). The frequency of single PLA-specific $CD4^+$ Tr1 cells ranges between 1 in 1'000 and 1 in 20'000 of the whole $CD4^+CD25^+$ T_{reg} cells. PLA T-cell epitope restriction pattern varies considerably from patient to patient, and at least 4 epitopes have been demonstrated (237,345). PLA-specific T cells demonstrated a clonality throughout the whole season. In two types of experiments, which investigated their T cell receptor clonality by PCR and flow cytometry, an immediate skew to IL-10 producing Tr1 type cells was *in vivo* observed. Similarly, IL-10-secreting allergen-specific T cells represented the predominant subset and were present at significantly higher frequency than IL-4- and IFN-γ-secreting T cells in food and aeroallergen sensitized individuals, who did not develop an allergic response (87).

Allergen-specific immunotherapy in humans has been used successfully to treat allergies by inducing responsive cells to secrete IL-10, and this is analogous to the studies of low-zone tolerance (346). The IL-10-producing $CD4^+$ Tr1-cell population that is induced by T cell epitope peptide immunotherapy and whole allergen immunotherapy induces specific Tr1 cells. Tr1 cells specific for a variety of antigens arise *in vivo*, but may also differentiate from naive $CD4^+$ T cells in the presence of IL-10 *in vitro* (108). The non-specific T cell suppressor activity of IL-10 and TGF-β has been consistently reported in experiments with high amounts of exogenously added suppressor cytokines (222). However, the present study demonstrates that Tr1 cells display antigen-specific suppressor activity in very low numbers. Depending on their frequency, the first T cell that contacts the APC may be very decisive for inducing or suppressing the generation of a specific immune response. Accordingly, if the first T cell to contact APC is a Tr1 cell, it may silence or regulate the maturation of APC. IL-10 down-regulates the antigen-presenting capacity such as HLA-DR expression, co-stimulatory molecules and several cytokines in dendritic cells and monocytes/macrophages (109). Differentiation of a distinct dendritic cell subset in the presence of IL-10 has been demonstrated, which induces tolerance through the generation of Tr1 cells (347).

Allergen-specific T cell tolerance utilizes multiple suppressor mechanisms. $CD4^+CD25^+$ T cells are the only lymphocyte subpopulation in both mice and humans that express CTLA-4 constitutively. The expression apparently correlates with the suppressor function of CTLA-4. As demonstrated in the present study for Tr1 cells,

the blocking of CTLA-4 activity reverses suppression in co-cultures of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells (105). Similarly, the treatment of mice, which are recipients of $CD4^+CD45RB^{low}$ T cells with CTLA-4-blocking agents abrogated the suppression of inflammatory bowel disease (348). These studies indicate that the engagement of CTLA-4 on the $CD4^+CD25^+$ T cells by antibody or by CD80/CD86 might lead to inhibition of the TCR-derived signals that are required for the induction of suppressor activity. PD-1 is an immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptor expressed upon T cell activation. PD-1-deleted mice develop autoimmune diseases, suggesting an inhibitory role for PD-1 in immune responses (106). Members of the B7 family, PD-ligand (L) 1 and PD-L2, are ligands for PD-1. PD-1/PD-L engagement on murine CD4 and CD8 T cells results in inhibition of proliferation and cytokine production. T cells stimulated with anti-CD3/PD-L1Fc-coated beads display dramatically decreased proliferation and IL-2 production (107).
The present study demonstrates that HR2 represents an essential receptor that participates in peripheral tolerance to allergens by induction of IL-10 and directly suppression of the proliferation of allergen-specific T cells. Differential patterns of HR expression on Th1 and Th2 cells determine reciprocal T cell responses following histamine stimulation. Human $CD4^+$ Th1 cells predominantly express HR1 and $CD4^+$ Th2 cells HR2, which results in their differential regulation by histamine (92). Histamine enhances Th1-type responses by triggering the HR1, whereas both Th1- and Th2-type responses are negatively regulated by histamine binding to HR2 (92). Histamine induces the production of IL-10 by DC (89), and enhances the suppressive activity of TGF-β on T cells (91). In the differentiation process of monocyte-derived dendritic cells, HR2 acts as a suppressive molecule for antigen-presentation capacity, suppresses IL-12 production and enhances IL-10 production (89,287). All of these immune tolerance promoting effects are mediated via HR2, which is relatively highly expressed on Th2 cells and suppresses IL-4 and IL-13 production and T cell proliferation (92).

Studies on T cell response to allergens in healthy individuals have demonstrated a wide range of immune response from no detectable response to involvement of active peripheral tolerance mechanisms (312). In a high number of healthy individuals, T cells do not show any proliferative response to allergens in PBMC cultures, which can be due to low frequency of specific T cells, due to lack of exposure or exposure below the threshold of sensitization dose. Active suppression against allergens by T_{reg} cells occurs in sensitized healthy individuals (87,224). In individuals, who show a detectable IgG4 response, a balanced allergen-specific immune response due to expansion of Tr1 cells has been demonstrated (87).

Collectively our results indicate that the control of Th2 and Th1 immune responses against naturally exposed harmless environmental antigens is mediated by Tr1 cells in humans. Effector (allergen-specific Th2) and suppressor (allergen-specific Tr1) T cells exist in both healthy and allergic individuals in certain amounts. Their ratio determines the development of a healthy or an allergic immune response. These data may explain the spontaneous development and spontaneous remission of allergic diseases. In addition to allergy, these mechanisms may have implications in autoimmunity, graft-versus-host disease, tumor cell growth, parasite survival and chronic infections.

Material and Methods

Study population and allergens. 10 beekeeper volunteers (mean age: 58 y, range 34-71 y, 9 male, 1 female, average bee keeping time 36 y; range 17-46 y) who reported that they do not use protection equipment and show cutaneous late-phase swelling response to bee stings in the beginning of each season. Physical examination and questionnaires have been performed, and heparinized peripheral venous blood samples have been taken. Recombinant phospholipase A_2 (PLA) and hyaluronidase (HYA) of honey bee venom (*Apis mellifera*), Bet v 1 of birch pollen (*Betula verrucosa*), rDer p 1 of house dust mite (*Dermatophagoides pteronyssinus*) were used. Purified protein derivative of mycobacterium bovis (PPD) and tetanus toxoid (TT) were used as control antigens. All of the allergens did not contain detectable amounts of LPS and were >99% pure.

Purification of allergen-specific IL-4-, IFN-γ- and IL-10-secreting cells. PBMC were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood and cells were washed three times and resuspended in RPMI 1640 medium supplemented as described (87). $2.5x10^7$ cells were stimulated with 0.3 μM antigens in 5 ml medium in 6-well plates in duplicates (Costar Corp., Cambridge, MA, USA). After 12 h of stimulation in humidified 5% CO_2, cells were harvested and labeled with 50 μg/ml of anti-IFN-γ/CD45, anti-IL-4/CD45 or anti-IL-10/CD45 Ab-Ab conjugates (Miltenyi Biotec, Bergish, Gladbach, Germany) for 10 min at a concentration of 10^8 cells/ml in ice cold RPMI 1640 medium (349). The cells were diluted with 37°C warm medium to a final concentration of 10^6 cell/ml and allowed to secrete and capture the respective cytokines for 45 min at 37°C. After capturing the secreted cytokines on their surface, cells were centrifuged at 300xg for 5 min at 4°C and resuspended at a concentration of 10^8 cells/ml in ice cold buffer containing 0.5% BSA and 5 mM EDTA (both from Sigma Chem. Co. St. Louis, MA) in PBS. The cells were then stained with 5 μg/ml PE-conjugated anti-IFN-γ, anti-IL-10 or anti-IL-4 for 10 min, at 4°C. Then, the cells were washed and resuspended in BSA-EDTA PBS (10^8 cells/ml) and magnetically labeled for 15 min at 4°C with 20 ml per 10^7 cells of anti-PE microbeads. After washing, labeled cells were purified by immunomagnetic separation (AutoMacs, Miltenyi Biotec, Bergish, Germany). The cells were counter-stained by FITC labeled anti-CD4 mAb (Immunotech, Marseilles, France) and analyzed in a flow cytometer (Epics XL, Coulter Corp. Hialeah, FL, USA) and their percentage in whole $CD4^+$ T cells and frequency was calculated.

Together with the quantitative cytokine mRNA and secreted cytokine profiles these data demonstrate that antigen-specific Tr1, Th1 and Th2 cells can be purified from human peripheral blood (Supplementary Fig. 6) and they are specific for the allergen that they are initially stimulated for the purification (Supplementary Fig. 7). The purity of allergen-specific $CD4^+$ cytokine-secreting cells was between 88-96%. The frequency of allergen stimulated and unstimulated cells was calculated by dividing the number of purified cytokine-secreting $CD4^+$ T cells by the initial number of $CD4^+$ T cells (Supplementary method).

T cell cultures. Allergen-specific cytokine-secreting T cells were used immediately after purification in all experiments. Allergen-specific T cell proliferative response was determined by stimulation of 2 x 10^5 PBMC alone or together with freshly purified allergen-specific cytokine-secreting T cells for 5 days with 0.3 µM allergens, 1 µg/ml tetanus toxoid and PPD in 200 ml medium in 96-well flat-bottom tissue culture plates in triplicates (123). Autologous 3000 rad irradiated PBMC were used as APC. Cells were pulsed with 1 mCi/well [^{3}H] thymidine (Du Pont / New England Nuclear, Boston MA) and incorporation of labeled nucleotide was determined after 8 h in a LKB beta plate reader (Wallax, Pharmacia Turku, Finland). Cell proliferation is additionally measured by carboxyfluoresceinsuccinimidyl ester (CFSE) labeling. Cells are labeled with CFSE (5 mM, Molecular Probes Europe, BV, Leiden, The Netherlands) and washed twice with medium before being subjected to the PLA stimulation. After 5 days, $CD4^+$ cells are counter stained with Pc5-labelled anti-CD4 and ECD-labelled anti-CD3 mAbs (BD PharMingen AG) and analysed by flow cytometry.

IL-10 was neutralized in cultures with 4 mg/ml anti-IL-10R mAb (DNAX Research Institute, Palo Alto, CA) (350). TGF-β was neutralized in cultures with 100 ng/ml recombinant human soluble TGF-β receptor II/Fc chimeric protein (R&D Systems, Wiesbaden, Germany) (222). Programmed death-1 (PD-1) activity was neutralized in cultures with 5 mg/ml anti-human PD-1 (Bioscience Insight Biotechnology Limited, Wembley, UK). Cytotoxic T-lymphocyte antigen 4 (CTLA-4) activity was neutralized with 5 mg/ml anti-CD152 F $(ab)^2$ (Ancell, Alexis Corporation, Lausen, Switzerland). The neutralizing activity of these approaches was controlled in titrated doses. Rabbit IgG, rat IgG, mouse IgG1 or bovine serum albumin (Coulter Corp.) served as control.

Flow cytometry and ELISPOT. PBMC of beekeepers before and 7 days after they received multiple bee stings were stimulated with PLA for 10 days. Intracellular cytokines were detected after anti-CD2, anti-CD3, and anti-CD28 mAb stimulation for 12 h. 2 mM monensin (Sigma Chem Co) was added during the last 10 h (98). The solid-phase sandwich ELISAs for IFN-γ, IL-4, IL-10 and IL-13 are performed in supernatants obtained at day 5 (98).

10^6/ml PBMC from 6 healthy donors were stimulated in 200 µl medium, 96-well flat bottom Enzyme-linked immunospot (ELISPOT) plates for 18 h (Euroclone Ltd., Milano, Italy). Locally produced IL-4, IFN-γ and IL-10 were captured by specific monoclonal antibodies. After cell lysis, trapped cytokine molecules were revealed by a secondary biotinylated detection antibody, which is recognised by streptavidin conjugated to alkaline phosphatase. Colored "purple" spots developed after substrate addition was determined (ImmunoSpot, Cellular Technology Ltd., Echterdingen, Germany). The number of spots determined in triplicates of unstimulated wells was subtracted from 0.3 µM PLA-stimulated wells. 18 h was found the optimal time for the determination of frequency of cytokine secreting cells, as it is the time point for highest cytokine secretion before T cell proliferation starts.

Quantitative real-time PCR. Immediately after purification, antigen-specific cytokine-secreting T cells were lysed with RNeasy lysis buffer and the RNA was isolated using the RNeasy mini kit (Qiagen, Hamburg, Germany) and eluted in 30 µl ddH_2O. Reverse transcription was performed with RevertAid M-MuLV Reverse Transcriptase, Random Hexamer Primers, dNTP Mix, Ribo Lock RNase Inhibitor (all from Fermentas Burlington, Canada). The PCR primers and probes were designed based on sequences reported in GenBank. Primers were: EF-1a forward primer 5`CTG AAC CAT CCA GGC CAA AT 3`, EF-1a reverse primer 5`GCC GTG TGG CAA TCC AAT 3`, IL-13 forward primer A 5` GCC CTG GAA TCC CTG ATC A 3`, IL-13 reverse primer A 5` GCT CAG CAT CCT CTG GGT CTT 3`, IFN-γ forward primer B 5` TCT CGG AAA CGA TGA AAT ATA CAA GTT AT 3`, IFN-γ reverse primer B 5` GTA ACA GCC AAG AGA ACC CAA AA 3`, IL-10 forward primer 5`GGC GCT GTC ATC GAT TTC TT 3`, IL-10 reverse primer 5`TTG GAG CTT ATT AAA GGC ATT CTT C 3`, TGF-β1 forward primer 5`AAA TTG AGG GCT TTC GCC TTA 3` and TGF-β1 reverse primer 5`GAA CCC GTT GAT GTC CAC TTG 3`, HR1 forward primer: 5'-TCT CGA ACG GAC TCA GAT ACC A-3', HR1 reverse primer: 5'-CCT GTG TTA GAC CCA CTC CTC AA-3', HR1 probe: FAM-ACA GAG ACA GCA CCA GGC AAA GGC AA-TAMRA; HR2 forward primer: 5'-GCT GGG CTA TGC

CAA CTC A-3', HR2 reverse primer: 5'-GGT GCG GAA GTC TCT GTT CAG-3', HR2 probe: FAM-CCC TGA ACC CCA TCC TGT ATG CTG C-TAMRA; HR4 forward primer, 5'-GGG TCT TGA AGA TTG TTA CTC TGA TG-3', HR4 reverse primer, 5'-CTA GAA TCA TTG GCC CAT TCA CT-3', HR4 probe: FAM-GCC AGC ACC CAA ACG GCC A-TAMRA; Foxp3 forward primer: 5'-CCC GGC CTT CCA CAG AA-3', Foxp3 reverse primer: 5'-CAC CCG CAC AAA GCA CTT G-3' (all were from Microsynth AG, Balgach, Switzerland). cDNAs were amplified using TaqMan® PCR mastermix (Applied Biosystems) according to the recommendations of the manufacturer in a total volume of 25µl in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Relative quantification was performed as described (351). All amplifications were carried out in duplicates.

TCR vb chain detection on CD4$^+$ T cells. 7 x 10^6 expanded PLA-specific T-cells from beekeepers, stimulated either with PLA or anti-CD2/3/28 before and after multiple bee stings or PBMC of healthy donors were blocked for 10 min. at room temperature with mouse serum (Dako AG, Baar, Switzerland) and subsequent stained for 10 min. with anti-CD3-FITC (Clone UCHT1, IgG1 mouse, Beckman Coulter, Lyon, Switzerland) and anti-CD4-PC5 (Clone 13B8.2, IgG1 mouse, Beckman Coulter) or matching isotype controls. After washing the assay was split into 22 samples and stained for 15 min. with PE-labeled anti-TCR Vb 1, 2, 3, 5.1, 5.2, 5.3, 7, 8, 9, 11, 12, 13.1, 13.6, 14, 16, 17, 18, 20, 21.3, 22, 23 mAbs or matching isotype controls (Beckman Coulter). The FACS measurement was performed on an Epics XL MCL (Beckman Coulter).

TcR clonality analysis. DNA was isolated with the DNA micro kit (Qiagen, Hilden, Germany) and clonality was determined using the IdentiClone T Cell Receptor Beta Gene (TCRB) Clonality Assay (*InVivo*Scribe Technologies, LLC, San Diego, CA, USA). This PCR based assay identifies clonal T cell receptor beta chain gene rearrangements. Multiple consensus DNA primers that target conserved genetic regions within the T cell receptor beta chain gene are used to detect these rearrangements. Specific primers in two assay tubes (A, B) target framework regions within the variable region and the joining region and the third assay tube targets the diversity and joining regions. The specimen control size ladder master mix targets multiple genes and generates a series of amplicons to ensure that the quality and quantity of the input DNA is sufficient for the test. The DNA was added to each of the four assay tubes together with AmpliTaq Gold DNA Polymerase (Applied Biosystems, Rotkreuz, Switzerland) and the DNA was amplified using a standard

program with a thermocycler (Eppendorf, Hamburg, Germany). A heteroduplex analysis was done to differentiate clonal and non-clonal PCR products. PCR products from tube A, B and C were denatured at 94°C for 5 minutes and immediately chilled at 4°C on an ice water bath for 60 min. This allows a re-annealing of the clonal strands, which results in a single band within a polyclonal background on the gel. Normal or polyclonal DNA produces amplicons of different size, which results in a smear. PCR products were separated on 6% Novex TBE Polyacrylamide gels (Invitrogen, Carlsbad, CA, USA), stained with ethidium bromide then scanned and analysed using a FUJIFILM BioImager Analyzer FLA-3000 with BASReader software (Fuji Photo Film Ltd., Tokyo, Japan), and the AIDA software (Raytest, Straubenhardt, Germany). Polyclonal and clonal control DNA was used to monitor the performance of the assay.

Statistical interpretation. Data are expressed as means $\pm$ SEM. Wilcoxon Rank Sum test and Mann-Whitney U test were used for statistical analysis.

Online supplemental material. Fig. S6 demonstrates that PLA-specific IL-4-, IFN-γ-, and IL-10-secreting T cells represent Th2-, Th1- and Tr1-like cells, respectively. Fig. S7 shows the antigen-specificity of purified cytokine-secreting T cells as determined by stimulation with the allergen that was originally used for stimulation before purification, and several control antigens in the presence of autologous APC. Supplementary method explains how the frequency of purified allergen-specific T cells is calculated.

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Online supplementary material

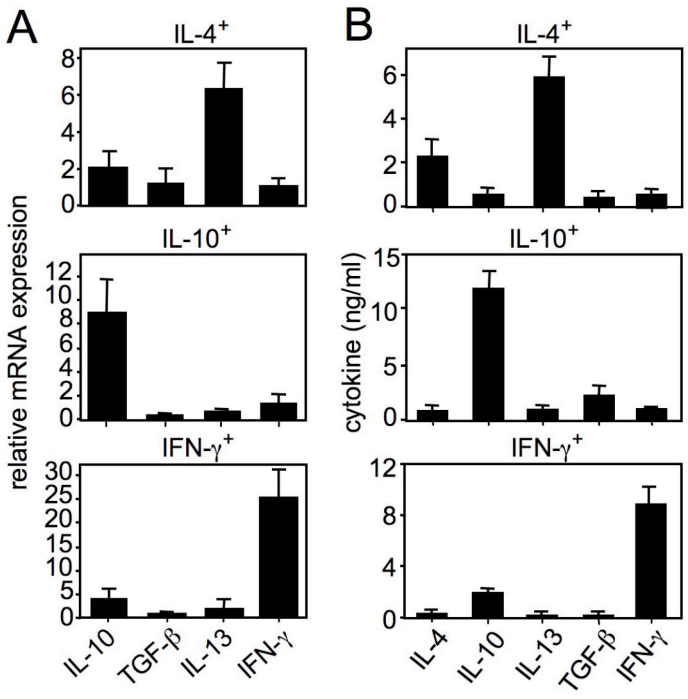

Figure S6

PLA-specific IL-4-, IFN-γ-, and IL-10-secreting T cells represent Th2-, Th1- and Tr1-like cells, respectively

$CD4^+$ T cells specific to bee venom PLA were isolated according to their IL-4, IFN-γ and IL-10 secretion profile. To confirm their cytokine profile, mRNA of IL-10, IL-13, IFN-γ and TGF-β were quantified immediately after isolation (Fig. S6A). Relative to the housekeeping gene EF-1α, IL-10-secreting T cells expressed significantly high IL-10 mRNA and IFN-γ-secreting T cells expressed significantly high IFN-γ mRNA. IL-13 mRNA was dominant in IL-4-secreting T cells. Same results were obtained in three independent experiments.

Purified allergen-specific cytokine-secreting T cells were expanded for 2 weeks in the presence of 1 nM doses of growth factors (IL-2 for IFN-γ-secreting T cells, IL-2 and IL-4 for IL-4-secreting T cells, IL-2 and IL-15 for IL-10-secreting T cells) (Novartis, Basel Switzerland). Quantification of their cytokine profile in supernatants by ELISA 72 hours after anti-CD3 and anti-CD28 mAb stimulation demonstrated that these subsets contain Th2-like (IL-4 and IL-13 high), Th1-like (IFN-γ high) and Tr1-like (IL-10 high) cells, respectively (all P <0.0001 compared to other subsets) (Fig. S6B). In addition, all three purified subsets consisted of some Th0 cells, which secrete both Th1 and Th2 cytokines as well as IL-10 and TGF-β.

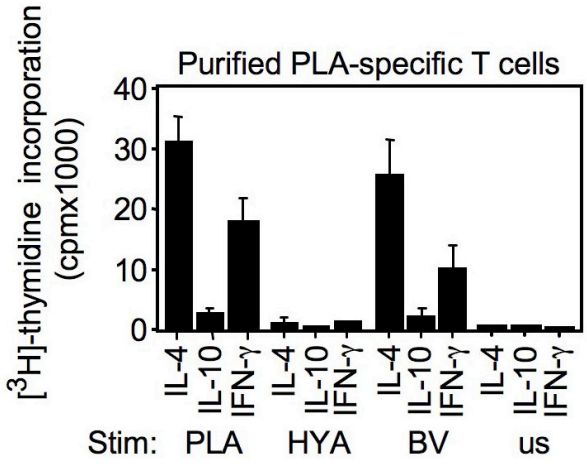

Figure S7

Antigen-Specificity of Purified Cytokine-Secreting T Cells

The antigen-specificity of purified cytokine-secreting T cells was determined by stimulation with the allergen that was originally used for stimulation before purification, and several control antigens in the presence of autologous APC. T cells purified by certain antigen stimulation did not show any cross-reactivity against control antigens. All three subsets purified by bee venom PLA stimulation responded to PLA and whole bee venom, but not to HYA as another bee venom major allergen. [^{3}H]-thymidine incorporation (TdR) was determined after 5 days. *:$p<0.001$. One representative of 3 different donors is shown.

Supplementary Method

Frequency calculation of purified allergen-specific T cells. The frequency of allergen stimulated and unstimulated cells was calculated by dividing the number of purified cytokine-secreting $CD4^+$ T cells by the initial number of $CD4^+$ T cells. To obtain the frequency of allergen-specific, cytokine-secreting T cells, the unstimulated cytokine-secreting $CD4^+$ T cell frequency was subtracted from allergen-stimulated cell number. Cells were counted by using 9 mm^2 area of a Neubauer counting chamber and trypan blue exclusion. The number of $CD4^+$ cells was calculated as follows as an example of PLA-specific, IL-10-secreting $CD4^+$ T cells. Allergen-stimulated cells: 47.4 x 10^6 (cell count from culture after 12 h) x 52.8 % (percentage of $CD4^+$ T cells) = 25.02 x 10^6 $CD4^+$ T cells at the start. Unstimulated cells: 47.1 x 10^6 (cell count from culture after 12 h) x 53.1 % (percentage of $CD4^+$ T cells) = 25.01 x 10^6 $CD4^+$ T cells at the start. Cells were counted and the number of purified $CD4^+$ IL-10-secreting T cells was calculated as follows. PLA-stimulated cells: 33'000 (cell

count after purification) x 92.4 % (percentage of purified $CD4^+$ $IL\text{-}10^+$ T cells) = 30'492 purified $CD4^+$ PLA-specific, IL-10-secreting T cells. Unstimulated cells: 11'000 (cell count after purification) x 88.4 % (percentage of purified $CD4^+$ $IL\text{-}10^+$ T cells) = 9'724 $CD4^+$ and IL-10-secreting T cells without any stimulation. Cell frequency was calculated by dividing purified $CD4^+$ IL-10-secreting T cell number by $CD4^+$ T cell number at the start of cytokine secretion assay. PLA-stimulated cells: 30'492 divided by $25.02 \times 10^6 = 0.001218$ (12.12 cells in 10'000). Unstimulated cells: 9'742 divided by $25.01 \times 10^6 = 0.000389$ (3.89 cells in 10'000). Final frequency of PLA-specific IL-10-secreting T cells is calculated by subtracting unstimulated $CD4^+$ IL-10-secreting cell frequency from PLA-specific $CD4^+$ IL-10-secreting cell frequency (0.001218 - 0.000389 = 0.000829). That means, 8.29 cells in 10'000 $CD4^+$ cells are PLA-specific, IL-10-secreting, Tr1-like cells. All of the frequency calculations shown in Figure 11A were performed as above.

6.3 Statement of contribution to publications

For the publication titled „Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors“, I performed all the experiments, except the methodology of ELISPOT.

For the publication titled „Clonal switch to IL-10-secreting type 1 T regulatory cells in high dose allergen exposure“ I contributed to the experiments depicted in figure 11A and 11B, 12, 13, 14A and 14B, and the supplementary figure 6.

7 Discussion

Allergic diseases result from the activation of the immune system and formation of IgE antibodies against normally innocuous environmental antigens. Th2 cells and their associated cytokines have been demonstrated to play a key role in the induction of IgE and in multiple other mechanisms of allergic inflammation. Alterations in the susceptible balance between allergen-specific Treg and Th2 cells is very pivotal in the development and also treatment of allergic diseases. During the last few years, there has been an increasing interest in discovering the effector mechanisms of Treg cells. Understanding such effects of Treg cells is important for the development of new therapeutical drugs to prevent and cure allergic diseases or autoimmune diseases.

7.1 Regulatory T cells play a role in the induction of IgG4 and suppression of IgE

The aim of the present study was to investigate the role of different subsets of Treg cells and toll-like receptors (TLRs) on IgE, IgG4 and IgA production. We used healthy individuals, because we investigated regulation of Ig isotype switch and production in normal conditions. We showed that regulatory T cells not only influence Th1 or Th2 cell responses, but that those cells also directly effect B cells. Freshly purified $CD4^+CD25^+$ Treg cells and IL-10-secreting Tr1 cells suppressed IgE and induced IgG4. Both regulatory T cell subsets also lowered IgE-secreting, and simultaneously augmented the IgG4-secreting plasma cell-frequency. In lymph nodes and tonsils, $CD4^+CD25^+Foxp3^+$ Treg cells are present in B cell areas where T-B cell interaction and humoral immune responses occur, and can directly influence B cell Ig production and CSR without the requirement to suppress Th cells. This is supported by studies, which show decreased Treg cell function or number in several immune mediated diseases in humans and mouse models with increased autoantibody production (310,311) and it has also been shown that elimination of $CD4^+CD25^+$ Treg cells results in development of various autoimmune diseases.

In healthy and allergic individuals, the Th1, Th2 and Tr1 type, allergen-specific subsets are found in different proportions (87). Normally, the Tr1 cell subset specific for common environmental allergens is dominant in healthy individuals (87,312) and a change in the fine balance between Th2 and Treg cells can lead to either allergy development or recovery. $CD4^+CD25^+$ Treg cells have a frequency of

~10-15% of $CD4^+$ T cells, while the incidence of Tr1 cells with a single allergen specificity is between 0.1 and 0.007% of $CD4^+$ T cells. In other words, one allergen-specific Tr1 cell can be found in 1'000 to 20'000 of the total $CD4^+$ T cell population. But Tr1 cells are not anergic and they can differentiate from naive $CD4^+$ T cells in the presence of IL-10 *in vitro* (108), and they have been shown to proliferate by IL-2, IL-4, IL-7, and IL-15 (87). Although suppression by Tr1 cells is antigen-specific, high numbers of Tr1 cells (at least 20'000 in 100'000 PBMCs) result in a non-specific suppressive activity on anti-CD3-stimulated T cells.

However, not only the ratio of different T cell subsets affects the outcome of an immune response, but also the ratio of certain antibody isotypes plays a very important role. Normally, allergic individuals have high levels of IgE and low amounts of IgG4 against a specific allergen. IgG4 has a low potential for inducing inflammation, because the IgG half-molecules can separate and pair again among IgG4, which leads to bi-specific antibodies that can not form large immune complexes (44,45). However, significant amounts of bispecific antibodies can be found only when two antigens, which induce high levels of IgG4 antibodies, are present at the same time in the body. Unless both antigens are present, the exchange process renders IgG4 antibodies monovalent (45). Furthermore, IgG4 is not able to activate the complement. In an allergen-specific immunotherapy study, in which well-defined recombinant allergen mixtures were used, all treated patients developed very strong allergen-specific IgG4 and also increased IgG1 antibody responses. Some patients, who were not initially sensitized to the major timothy grass pollen allergen Phl p 5 (Phleum pratense), developed strong specific IgG4, but not IgE antibody responses to that allergen (317).

Allergen-specific IgG4 may be directed against the same epitopes as IgE, resulting in direct competition for allergen binding and a “blocking” effect. Approximately after 3 months of SIT, IgG4 antibodies reach sufficiently high levels or affinities to compete with the binding of IgE to allergens (217), and therefore, not only IgE-mediated allergen presentation to $CD4^+$ T cells is prevented, but also histamine release from mast cells and basophils might be inhibited since the allergen is captured before reaching the effector cell-bound IgE (352). Kepley et al. showed that this reduced histamine release could be caused by a direct inhibition of the allergen-IgE interaction or by coaggregation of the inhibitory FcγRIIB and FcεRI (353).

Until recently, mast cells were thought to be the most important target cell that needed protection from IgE-mediated allergen triggering. However, the enormous potential of allergen-specific IgE antibodies in allergen presentation to T cells must also be taken into consideration (217,354,355). Therefore, competition among IgE

and IgG4 at the level of antigen-presenting cell and the induction of IgG4 during SIT also dampens the T-cell dependent late-phase allergic reaction. Through inhibition of IgE-mediated allergen presentation, involvement of B cells as APC is reduced and the threshold for the activation of allergen-specific Th2 cells is enhanced. T cells are therefore activated through the presentation of the allergen by professional APCs such as DCs or macrophages, which requires higher allergen concentrations, favoring a Th1 response (356). During SIT, additional treatment with anti-IgE prevents the IgE-mediated allergen presentation and changes the sensitivity of the immune system for the allergens (357,358).

IL-10 is a potent suppressor of both total and allergen-specific IgE, while it simultaneously increases IgG4 production (129). Thus, IL-10 not only generates tolerance in T cells, it also regulates specific isotype formation and skews the specific response from an IgE to an IgG4 dominated phenotype. The healthy immune response to Der p 1 demonstrated increased specific IgA and IgG4, small amounts of IgG1 and almost undetectable IgE antibodies in serum (222). House dust mite-SIT did not significantly change specific IgE levels after 70 days of treatment, however, a significant increase in specific IgA, IgG1 and IgG4 was observed (222). Most probably the decrease in IgE/IgG4 ratio during allergen-SIT is a feature of skewing an allergen-specific Th2 to a Treg cell predominance. However, although Treg cell generation happens within days, a significant decrease in IgE/IgG4 ratio occurs after several months. The reason for the long-time gap between the change in T cell subsets, but not IgE/IgG4 levels is not easily explainable by the half-life of antibodies. In this context, the role of bone marrow-residing IgE-producing plasma cells with very long lifespan remains to be investigated (318). It has also been suggested that rather affinity and epitope specificities of the induced IgG4 than the concentration should be analysed, since these features might be more relevant for the clinical effect of the antibodies (359).

In patients allergic to bee venom, intramuscular or intravenous application of plasma from hyperimmune beekeepers with high levels of specific IgG showed a beneficial effect in a following allergen exposure (360,361). Passive administration of polyclonal human allergen-specific IgG could be a new way of treating allergy, however, it will probably not be as effective as SIT, because induction of an altered cytokine profile in T cells can not be realised. But, since IgG antibodies have a long half-life, two to three injections could be enough to obtain a protective effect during one pollen season.

If the mechanism by which regulatory T cells act on B cells consists of physical cell-to-cell contact via ICAM-1, cell surface-bound TGF-β (130), CTLA-4

(322), GITR (323), PD-1 (324) and OX40 (CD134) (325), or, as very recently shown, via cyclic adenosine monophosphate and gap junctions (326), or if it is more an autocrine mechanism with IL-10 and TGF-β as soluble mediators, is not known yet. It appears however, that this antibody isotype regulation is partially dependent on IL-10 as demonstrated in our inhibition experiments with anti-IL-10 and anti-IL-10 receptor-antibodies and the transwell experiments.

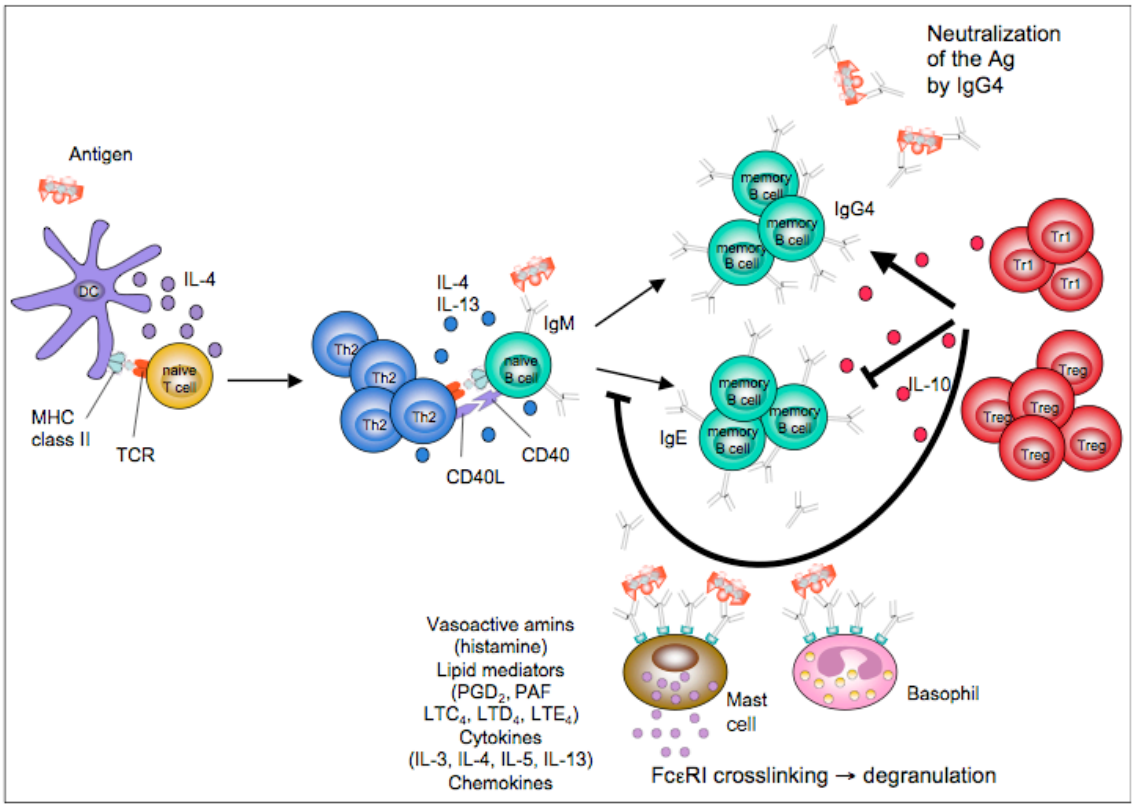

Figure 17. Antigen-presenting, IL-4 producing DCs prime naive T cells to differentiate to Th2 cells, which produce high amounts of IL-4 and IL-13. Those Th2 cytokines induce antibody isotype switching to IgE and IgG4 antibodies in naive B cells. IgG4 plays an important role in neutralizing antigens, while IgE binds to FcεRI on mast cells and basophils. Antigen-binding and cross-linking of FcεRI leads to mast cell and basophil degranulation and release of vasoactive amins, lipid mediators, cytokines and chemokines, which subsequently elicit inflammatory effector responses. One task of both, Tr1 cells and $CD4^+CD25^+$ Treg cells is to induce the non-inflammatory IgG4 antibodies, but to suppress isotype switch to and production of inflammatory IgE antibodies.

7.2 IgA production is regulated by the innate immune system

Furthermore, we demonstrated that direct TLR7 or TLR9 stimulation on B cells plays an important role for enhancing IgA production, while induction of IgA by Treg cells is very limited. This is a crucial finding in human Ig isotype regulation. In our experiments, IgA production is neither influenced by IL-10 nor by IL-10-secreting Tr1 cells nor by $CD4^+CD25^+$ Treg cells. This antibody isotype is particularly regulated by activation of the innate immune system. Addition of TLR7 or 9 agonists to PBMC cultures highly upregulated IgA production and secretion, whereas T cell-help to B cells seems not to be necessary, since Tr1 cells and $CD4^+CD25^+$ Treg cells did not have any effect on IgA production. Recent evidence indicates that TLR stimulation on B cells can control immunoglobulin isotype switch. In mice, TLR9 and MyD88 are required for class switching to the pathogenetic IgG2a and IgG2b isotypes, but not for the development of IgM autoantibodies (186). Furthermore, CpG, but not LPS, upregulates T-bet expression in B cells and induces IgG2a, whereas it decreases IgG1 and IgE production (187,188). In humans, IL-10 upregulates TLR9 expression in B cells and TLR9 stimulation by CpG-DNA, in association with IL-10, initiates germline immunoglobulin heavy chain constant region $C\gamma1$, $C\gamma2$ and $C\gamma3$ gene transcription, but not $C\gamma4$ or $C\varepsilon$, and $C\alpha$ has not been measured (190). Besides having a direct effect on B cells, TLR agonists can affect B cell differentiation and isotype switch indirectly through activation of DCs and subsequent release of IFN-α and IL-6 (191). Recently, it has been shown in mice and human, that gut DCs induce IgA class switch by synergistic effects of retinoic acid, IL-6 and IL-5 (195), however, the role of TLRs in DC mediated IgA induction remains to be elucidated. Interestingly, Lanzavecchia et al. suggest that extensive B cell proliferation, isotype switch and differentiation to Ig-secreting plasma cells require three signals: BCR triggering, T cell help and TLR stimulation (184). Zinkernagel et al. showed, that in mice, intestinal secretory IgA is produced by a T cell-independent and follicular organized lymphoid tissue-independent B cell subpopulation and derived largely from B1 peritoneal cells. This IgA production required the presence of the intestinal microflora and is therefore antigen-driven and does not simply reflect the presence of natural antibodies (177).

To increase the efficacy of allergen-SIT, usage of TLR7/9 triggering substances as an adjuvant together with recombinant proteins may have a beneficial effect in allergen-specific immunotherapy. In this way, the blocking antibody isotype IgA is expected to increase simultaneously and support or even synergize the advantageous effects of IgG4 (218,319). Furthermore, CpG ODN used as adjuvants

for SIT, have been shown to skew the pathogenic Th2 immune profile towards a Th1 profile (362). In another study, IFN-α-, IFN-γ-, LPS- or CD40L-stimulated human DCs up-regulated BAFF and APRIL, which induced together with IL-4, IL-10 or TGF-β CD40-independent class-switch recombination to Cγ, Cα or Cε in IgD^+ B cells (192). In addition, TLR ligands and APRIL were shown to synergize to further enhance IgA2 class switching (320). Moreover, it has been demonstrated that inducible-nitric-oxide-synthase (iNOS) induces TGF-βRII on B cells and controls T-cell-dependent IgA class-switch recombination. Mucosa-associated lymphoid tissue (MALT) DCs express iNOS in response to the recognition of commensal bacteria by TLRs, which in turn leads to the production of BAFF and APRIL and subsequent T-cell-independent IgA class-switch recombination in B1 cells (321). In the present study, we showed that stimulation of TLR7 and 9 highly induced total IgA in healthy individuals. It seems that IgA production is strongly dependent on the innate immune system.

7.3 Induction of peripheral tolerance depends on a clonal switch towards IL-10-secreting Tr1 cells

Discrimination between harmless and pathological antigens to prevent unnecessary and self-destructive immune responses is one of the most demanding task of the immune system (341,363). To get more insight into the mechanisms of the induction of peripheral tolerance, we used beekeepers as a human model to examine how high amounts of soluble protein antigens, which enter the body subcoutaneously via bee-stings, are tolerated. The cytokine profile of PLA-specific memory T cells show an IL-10 and IFN-γ predominant profile together with barely detectable IL-4 and IL-13 beyond the beekeeping season. Upon bee venom exposure, an immediate switch of allergen-specific T cells from Th1 and Th2 towards Tr1 cells can be observed within a few days and, very interestingly, these sequential events are repeated every year. At the end of every beekeeping season when there is no exposure to the allergen anymore, peripheral T cell response returns within 2 to 3 months to the same levels as before exposure. The simoultaneous changes in cutaneous late-phase response, T cell proliferation and the cytokine profile suggest that T cell tolerance induced by an *in vivo* switch to Tr1-like IL-10-secreting cells is short-lived and dependent on antigen exposure. There is so far no report about *in vivo* turnover and lifespan of Tr1 cells in humans, but our data are in agreement with the *in vivo* doubling time of

FoxP3^{+}CD25^{hi}CD4^{+} T cells, which was demonstrated to be relatively short (8 days) in resting conditions compared to FoxP3^{-}CD25^{-}CD4^{+} T cells (24 days) (342).

Another major finding of the beekeeper study shows *in vivo* clonal switch of allergen-specific IL-4-producing and IFN-γ-producing T cells towards IL-10-producing T cells. PLA-specific T cells are able to proliferate persistently throughout consecutive years in beekeepers demonstrating a biphasic T cell response with substantial peripheral T cell tolerance upon high dose allergen exposure. The long term PLA-specific T cell repertoire has been maintained by an average of 42% IFN-γ-secreting T cells, 43% IL-10-secreting T cells and 15 % IL-4-secreting T cells. Immediately, after receiving bee-stings, the repertoire changed and consisted of 80% IL-10-secreting T cells, 14% IFN-γ-secreting T cells and 6% IL-4-secreting T cells. The mechanisms underlying maintenance of cytokine profiles in differentiated effector and memory T cell subsets in humans is still not fully known. It has been demonstrated that human T cells exhibit plasticity in the differentiation program. Lineage-committed effector T cells and antigen-specific central memory T cells keep their ability to change their cytokine and chemokine receptor profiles in response to the expression of opposing transcription factors (343,344).

PLA T-cell epitope restriction pattern is different in each individual, and at least 4 epitopes have been demonstrated to bind efficiently to human leukocyte antigens (HLA) II (237,345). In the beekeepers, PLA-specific T cells demonstrated clonality throughout the whole beekeeping season. We investigated their TCR clonality by PCR and flow cytometry, and could observe an immediate skew to IL-10-producing Tr1 type cells *in vivo*. In individuals who were sensitized to food and aeroallergens and did not develop an allergic response, IL-10-secreting allergen-specific T cells represented the predominant subset compared to IL-4- and IFN-γ-secreting T cells (87).

In many studies, high amounts of exogenously added IL-10 and TGF-β provoked non-specific T cell suppressor activity (222). In the present study however, we showed that Tr1 cells display antigen-specific suppressor activity in very low numbers. Depending on their frequency, the first T cell that contacts the APC may be very decisive for inducing or suppressing the generation of a specific immune response. In the case of a Tr1 cell, the maturation of the APC may be arrested, since IL-10 down-regulates the antigen-presenting capacity such as HLA-DR expression, co-stimulatory molecules and several cytokines in DCs and monocytes/macrophages (109). Differentiation of a CD11c^{low}CD45RB^{high} DC subset in the presence of IL-10 has been demonstrated, which induces tolerance by generating Tr1 cells (347).

It is still not clearly known which suppressor mechanisms allergen-specific Treg cells use to induce tolerance. CTLA-4 has been shown to be constitutively and specifically expressed on both human and mice $CD4^+CD25^+$ T cells, and the expression apparently correlates with the suppressor function of CTLA-4. The present and also previous studies demonstrated that blocking of CTLA-4 reverses the suppressor capacity of Tr1 and $CD4^+CD25^+$ T cells (105). Transfer of $CD4^+CD45^{low}CD25^+$ into mice with colitis and subsequent treatment with CTLA-4-blocking agents reduced the suppression of the disease (348). These studies indicate that the engagement of CTLA-4 on the $CD4^+CD25^+$ T cells by antibody or by CD80/CD86 might lead to inhibition of the TCR-derived signals that are required for the induction of suppressor activity. We also found that PD-1, which is an immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptor expressed upon T cell activation, seems to play an important role in the induction of tolerance. PD-1-deleted mice develop autoimmune diseases, suggesting an inhibitory role for PD-1 in immune responses (106).

Another major finding of the present study is that HR2 represents a further essential receptor that participates in peripheral tolerance to allergens. We investigated the regulation of HRs on allergen-specific T cells and HR2 showed a significant increase exclusively in allergen/PLA-stimulated (IL-4-secreting) Th2 cells during the beekeeping season. Furthermore, histamine upregulated IL-10 production in Th2 cells via HR2 triggering and thereby converted them into Tr1 cells. Subsequently, the allergen/PLA-specific proliferation of $CD3^+CD4^+$ T cells was significantly reduced and could be reversed by addition of anti-IL-10R blocking antibody or ranitidine, a HR2 antagonist.

Most PBMC cultures from healthy individuals do not show any T cell proliferative response to allergens. Reasons for that could be low frequency of specific T cells, lack of exposure or exposure below the threshold of sensitization dose. In sensitized healthy individuals, active suppression against allergens by Treg cells occurs (87,224). In individuals, who show a detectable IgG4 response, a balanced allergen-specific immune response due to expansion of Tr1 cells has been demonstrated (87). We suggest that Th2 and Th1 immune responses against naturally exposed harmless environmental antigens are controlled by Tr1 cells in humans. Effector (allergen-specific Th2) and suppressor (allergen-specific Tr1) T cells exist in both healthy and allergic individuals in certain amounts. Their ratio determines the development of a healthy or an allergic immune response. Our data may explain the spontaneous development and spontaneous remission of allergic diseases. In addition to allergy, these mechanisms may have implications in

autoimmunity, graft-versus-host disease, tumor cell growth, parasite survival and chronic infections.

7.4 Conclusion and outlook

We conclude that peripheral tolerance utilizes multiple mechanisms to suppress allergic inflammation. Apparently, Treg cells contribute to the control of allergen-specific immune responses in several ways: I) Suppression of antigen-presenting cells that support the generation of effector Th2 and Th1 cells; II) Suppression of Th2 and Th1 cells; III) Suppression of mast cells, basophils and eosinophils; IV) Interaction with resident tissue cells and remodeling (88). In addition to the above mechanisms, the present study demonstrates suppression of the inflammatory antibody isotype IgE and induction of the non-inflammatory antibody isotype IgG4 by Tr1 and $CD4^+CD25^+$ Treg cells. Furthermore, we got new insights into the lifespan and allergen-dependence of allergen-specific peripheral T cell tolerance, as well as clonal switch of allergen-specific IL-4-producing and IFN-γ-producing T cells towards IL-10-producing Tr1 cells. Besides IL-10 and TGF-β, Tr1 cells seem to require CTLA-4 and PD-1 for suppressing antigen-specific T cell proliferation. And finally, after high dose allergen exposure, HR2 is highly upregulated and plays a further pivotal role in the induction of peripheral tolerance.

The clinical relevance of Treg cells is of great interest. Generation of Treg cells or the increase of their suppressive capacity by drugs, cytokines or costimulatory molecules is an important target not only for the application in allergy and asthma, but also for transplantation and autoimmunity. However, it must be considered that Treg cells are not always responsible for a healthy immune response, since they can also induce chronicity of infections and tumor tolerance. Cellular therapy with antigen-specific Treg cells is another promising approach, since maintenance of long-term immune modulation and avoiding of general immunosuppression could be realized.

8 Curriculum vitae

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Examinations and courses during PhD

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Prof. Dr. K. Blaser, PhD,
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Presentations given in SIAF

Progress report	**Journal cub**
13 October 2004	24 May 2004
19 April 2005	9 February 2005
20 September 2005	6 September 2005
30 May 2006	20 June 2006
24 October 2006	12 September 2006
21 June 2007	20 February 2007
29 October 2007	19 September 2007
26 February 2008	19 February 2008

Congress Attendance

2th EAACI GA^{2}LEN Davos Meeting, 'Basic Immunology Research in Allergy and Clinical Immunology', February 2004. Davos, Switzerland

3th EAACI GA^{2}LEN Davos Meeting, 'Basic Immunology Research in Allergy and Clinical Immunology', February 2005. Davos, Switzerland
Poster

XVIIth meeting of the swiss immunology PhD students, March 2005. Schloss Wolfsberg, Ermatingen, Switzerland
Poster

4th EAACI GA^{2}LEN Davos Meeting, 'Basic Immunology Research in Allergy and Clinical Immunology', February 2006. Grainau, Garmisch-Patenkirchen, Germany
Oral presentaion

XVIIIth meeting of the swiss immunology PhD students, March 2006. Schloss Wolfsberg, Ermatingen, Switzerland
Oral presentation

Annual Congress SGAI-SSAI/Spring Meeting SGDV-SSDV, April 2006. Zurich, Switzerland
Poster

5th EAACI GA^{2}LEN Davos Meeting, 'Basic Immunology Research in Allergy and Clinical Immunology', February 2007. Davos, Switzerland
Poster

Keystone Symposia, Immunologic Memory, March 2007. Santa Fe, New Mexico, USA
Poster

XIXth meeting of the swiss immunology PhD students, March 2007. Schloss Wolfsberg, Ermatingen, Switzerland

Oral presentation
World Immune Regulation Meeting I, Special focus on regulatory cells, April 2007. Davos, Switzerland
Poster

World Immune Regulation Meeting II, Special focus on regulatory cells, March 2008. Davos, Switzerland
Poster

Publications

F. Meiler, M. Zimmermann, C.A. Akdis und M. Akdis. Regulatorische T-Zellen in der normalen Immunantwort und in der allergenspezifischen Immuntherapie. Allergologie, 2005, 28/6: 230-237

M. Zimmermann, F. Meiler, M. Akdis und C.A. Akdis. Immunologische Mechanismen der unterschiedlichen allergischen Phänotypen. Allergologie, 2005, 28/6: 219-226

F. Meiler, M. Zimmermann, K. Blaser, C.A. Akdis and M. Akdis. T-cell subsets in the pathogenesis of human asthma. Curr Allergy Asthma Rep., 2006, 6(2): 91-6

F. Meiler, S. Klunker, M. Zimmermann, C.A. Akdis and M. Akdis. Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors.
(Allergy 2008, in press)

F. Meiler, J. Zumkehr, S. Klunker, B. Rückert, C. A. Akdis, M. Akdis
Clonal switch to IL-10-secreting type 1 T regulatory cells in high dose allergen exposure.
(submitted)

9 References

1. A.G.N. The Late Baron Shibasaburo Kitasato. Can Med Assoc J 1931;**25**
2. Winau F, Westphal O, Winau R. Paul Ehrlich--in search of the magic bullet. Microbes Infect 2004;**6**:786-789.
3. Silverstein AM. Cellular versus humoral immunology: a century-long dispute. Nat Immunol 2003;**4**:425-428.
4. Van Epps HL. Michael Heidelberger and the demystification of antibodies. J Exp Med 2006;**203**:5.
5. The Linus Pauling Papers: How Antibodies and Enzymes Work.
6. Silverstein AM. Labeled antigens and antibodies: the evolution of magic markers and magic bullets. Nat Immunol 2004;**5**:1211-1217.
7. Edelman GM GJA. The nature of Bence-Jones proteins. Chemical similarities to polypeptide chains of myeloma globulins and normal gamma-globulins. J Exp Med 1962;**116:207-27**
8. Raju TN. The Nobel chronicles. 1972: Gerald M Edelman (b 1929) and Rodney R Porter (1917-85). Lancet 1999;**354**:1040.
9. Tomasi TB. The discovery of secretory IgA and the mucosal immune system. Immunol Today 1992;**13**:416-418.
10. Preud'homme JL, Petit I, Barra A, Morel F, Lecron JC, Lelievre E. Structural and functional properties of membrane and secreted IgD. Mol Immunol 2000;**37**:871-887.
11. Johansson SG. The discovery of immunoglobulin E. Allergy Asthma Proc 2006;**27**:S3-6.
12. Romagnani S. Lymphokine production by human T cells in disease states. Annu Rev Immunol 1994;**12**:227-257.
13. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996;**17**:138-146.
14. Rincon M, Anguita J, Nakamura T, Fikrig E, Flavell RA. Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. J Exp Med 1997;**185(3)**:461-469.
15. Corry DB. IL-13 in allergy: home at last. Curr Opin Immunol 1999;**11(6)**:610-614.
16. Akdis CA, Blaser K, Akdis M. Apoptosis in tissue inflammation and allergic disease. Curr Opin Immunol 2004;**16**:717-723.

17. Trautmann A, Akdis M, Kleemann D, et al. T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. J Clin Invest 2000;**106**:25-35.
18. Trautmann A, Schmid-Grendelmeier P, Kruger K, et al. T cells and eosinophils cooperate in the induction of bronchial epithelial cell apoptosis in asthma. J Allergy Clin Immunol 2002;**109**:329-337.
19. Trautmann A, Akdis M, Brocker EB, Blaser K, Akdis CA. New insights into the role of T cells in atopic dermatitis and allergic contact dermatitis. Trends Immunol 2001;**22**:530-532.
20. Trautmann A, Akdis M, Schmid-Grendelmeier P, et al. Targeting keratinocyte apoptosis in the treatment of atopic dermatitis and allergic contact dermatitis. J Allergy Clin Immunol 2001;**108**:839-846.
21. Yssel H, Groux H. Characterization of T cell subpopulations involved in the pathogenesis of asthma and allergic diseases. Int Arch Allergy Immunol 2000;**121**:10-18.
22. El Biaze M, Boniface S, Koscher V, et al. T cell activation, from atopy to asthma: more a paradox than a paradigm. Allergy 2003;**58(9)**:844-853.
23. Trautmann A, Altznauer F, Akdis M, et al. The differential fate of cadherins during T-cell-induced keratinocyte apoptosis leads to spongiosis in eczematous dermatitis. J Invest Dermatol 2001;**117**:927-934.
24. Abbas AK, Lichtman AH. Cellular and Molecular Immunology. Fifth ed. Philadelphia: Saunders; 2003.
25. Goding JW. Allotypes of IgM and IgD receptors in the mouse: a probe for lymphocyte differentiation. Contemp Top Immunobiol 1978;**8**:203-243.
26. Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. J Exp Med 1998;**188**:1679-1689.
27. Borghesi L, Milcarek C. From B cell to plasma cell: regulation of V(D)J recombination and antibody secretion. Immunol Res 2006;**36**:27-32.
28. Goldsby RA, Kindt TJ, Osborne BA, Kuby J. Immunology. Fifth ed. New York: Freeman; 2003.
29. Black SJ, van der Loo W, Loken MR, Herzenberg LA. Expression of IgD by murine lymphocytes. Loss of surface IgD indicates maturation of memory B cells. J Exp Med 1978;**147**:984-996.

30. Hayakawa K, Ishii R, Yamasaki K, Kishimoto T, Hardy RR. Isolation of high-affinity memory B cells: phycoerythrin as a probe for antigen-binding cells. Proc Natl Acad Sci U S A 1987;**84**:1379-1383.
31. Market E, Papavasiliou FN. V(D)J recombination and the evolution of the adaptive immune system. PLoS Biol 2003;**1(1)**:E16.
32. Roux KH. Immunoglobulin structure and function as revealed by electron microscopy. Int Arch Allergy Immunol 1999;**120**:85-99.
33. Diaz M, Casali P. Somatic immunoglobulin hypermutation. Curr Opin Immunol 2002;**14**:235-240.
34. Pier GB, Lyczak JB, Wetzler LM. Immunology, Infection, and Immunity. ASM Press; 2004.
35. Fuleihan R, Ramesh N, Geha RS. Role of CD40-CD40-ligand interaction in Ig-isotype switching. Curr Opin Immunol 1993;**5**:963-967.
36. Van Kooten C, Banchereau J. CD40-CD40 ligand: a multifunctional receptor-ligand pair. Adv Immunol 1996;**61**:1-77.
37. Stavnezer J. Antibody class switching. Adv Immunol 1996;**61**:79-146.
38. Honjo T, Kinoshita K, Muramatsu M. Molecular mechanism of class switch recombination: linkage with somatic hypermutation. Annu Rev Immunol 2002;**20**:165-196.
39. Durandy A. Activation-induced cytidine deaminase: a dual role in class-switch recombination and somatic hypermutation. Eur J Immunol 2003;**33**:2069-2073.
40. Casali P, Zan H. Class switching and Myc translocation: how does DNA break? Nat Immunol 2004;**5**:1101-1103.
41. Manis JP, Tian M, Alt FW. Mechanism and control of class-switch recombination. Trends Immunol 2002;**23(1)**:31-39.
42. Lieber MR, Yu K, Raghavan SC. Roles of nonhomologous DNA end joining, V(D)J recombination, and class switch recombination in chromosomal translocations. DNA Repair (Amst) 2006;**5**:1234-1245.
43. Stavnezer J, Amemiya CT. Evolution of isotype switching. Semin Immunol 2004;**16**:257-275.
44. Aalberse RC, Schuurman J. IgG4 breaking the rules. Immunology 2002;**105**:9-19.
45. van der Neut Kolfschoten M, Schuurman J, Losen M, et al. Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. Science 2007;**317(5844)**:1554-1557.

46. Amigorena S, Bonnerot C, Drake JR, et al. Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B lymphocytes. Science 1992;**256(5065)**:1808-1812.
47. Muta T, Kurosaki T, Misulovin Z, Sanchez M, Nussenzweig MC, Ravetch JV. A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling. Nature 1994;**368(6466)**:70-73.
48. Young JD, Ko SS, Cohn ZA. The increase in intracellular free calcium associated with IgG gamma 2b/gamma 1 Fc receptor-ligand interactions: role in phagocytosis. Proc Natl Acad Sci U S A 1984;**81(17)**:5430-5434.
49. Anderson CL, Shen L, Eicher DM, Wewers MD, Gill JK. Phagocytosis mediated by three distinct Fc gamma receptor classes on human leukocytes. J Exp Med 1990;**171(4)**:1333-1345.
50. Titus JA, Perez P, Kaubisch A, Garrido MA, Segal DM. Human K/natural killer cells targeted with hetero-cross-linked antibodies specifically lyse tumor cells in vitro and prevent tumor growth in vivo. J Immunol 1987;**139(9)**:3153-3158.
51. Pearse RN, Kawabe T, Bolland S, Guinamard R, Kurosaki T, Ravetch JV. SHIP recruitment attenuates Fc gamma RIIB-induced B cell apoptosis. Immunity 1999;**10(6)**:753-760.
52. Hamre R, Farstad IN, Brandtzaeg P, Morton HC. Expression and modulation of the human immunoglobulin A Fc receptor (CD89) and the FcR gamma chain on myeloid cells in blood and tissue. Scand J Immunol 2003;**57(6)**:506-516.
53. Monteiro RC, Van De Winkel JG. IgA Fc receptors. Annu Rev Immunol 2003;**21**:177-204.
54. Pleass RJ, Lang ML, Kerr MA, Woof JM. IgA is a more potent inducer of NADPH oxidase activation and degranulation in blood eosinophils than IgE. Mol Immunol 2007;**44(6)**:1401-1408.
55. Takigawa M, Tamamori T, Horiguchi D, et al. Fc epsilon receptor II/CD23-positive lymphocytes in atopic dermatitis. I. The proportion of Fc epsilon RII+ lymphocytes correlates with the extent of skin lesion. Clin Exp Immunol 1991;**84(2)**:275-282.
56. Bujanowski-Weber J, Brings B, Knoller I, Pfeil T, Konig W. Expression of low-affinity receptor for IgE (Fc epsilon RII, CD23) and IgE-BF (soluble CD23) release by lymphoblastoid B-cell line RPMI-8866 and human peripheral lymphocytes of normal and atopic donors. Immunology 1989;**66(4)**:505-511.
57. Maurer D, Fiebiger E, Reininger B, et al. Expression of functional high affinity immunoglobulin E receptors (Fc epsilon RI) on monocytes of atopic individuals. J Exp Med 1994;**179(2)**:745-750.

58. Tunon-De-Lara JM, Redington AE, Bradding P, et al. Dendritic cells in normal and asthmatic airways: expression of the alpha subunit of the high affinity immunoglobulin E receptor (Fc epsilon RI -alpha). Clin Exp Allergy 1996;**26(6)**:648-655.
59. Gould HJ, Sutton BJ, Beavil AJ, et al. The biology of IGE and the basis of allergic disease. Annu Rev Immunol 2003;**21**:579-628.
60. Maurer D, Ebner C, Reininger B, et al. The high affinity IgE receptor (Fc epsilon RI) mediates IgE-dependent allergen presentation. J Immunol 1995;**154(12)**:6285-6290.
61. Lantz CS, Yamaguchi M, Oettgen HC, et al. IgE regulates mouse basophil Fc epsilon RI expression in vivo. J Immunol 1997;**158(6)**:2517-2521.
62. Yamaguchi M, Lantz CS, Oettgen HC, et al. IgE enhances mouse mast cell Fc(epsilon)RI expression in vitro and in vivo: evidence for a novel amplification mechanism in IgE-dependent reactions. J Exp Med 1997;**185(4)**:663-672.
63. Saini SS, Klion AD, Holland SM, Hamilton RG, Bochner BS, Macglashan DWJ. The relationship between serum IgE and surface levels of FcepsilonR on human leukocytes in various diseases: correlation of expression with FcepsilonRI on basophils but not on monocytes or eosinophils. J Allergy Clin Immunol 2000;**106(3)**:514-520.
64. MacGlashan DWJ, Bochner BS, Adelman DC, et al. Down-regulation of Fc(epsilon)RI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody. J Immunol 1997;**158(3)**:1438-1445.
65. MacGlashan DJ, McKenzie-White J, Chichester K, et al. In vitro regulation of FcepsilonRIalpha expression on human basophils by IgE antibody. Blood 1998;**91(5)**:1633-1643.
66. Prussin C, Griffith DT, Boesel KM, Lin H, Foster B, Casale TB. Omalizumab treatment downregulates dendritic cell FcepsilonRI expression. J Allergy Clin Immunol 2003;**112(6)**:1147-1154.
67. Kay AB. Allergy and allergic diseases. First of two parts. N Engl J Med 2001;**344(1)**:30-37.
68. Kay AB. Allergy and allergic diseases. Second of two parts. N Engl J Med 2001;**344(2)**:109-113.
69. Yazdanbakhsh M, van den Biggelaar A, Maizels RM. Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. Trends Immunol 2001;**22(7)**:372-377.
70. Chippaux JP, Goyffon M. Venoms, antivenoms and immunotherapy. Toxicon 1998;**36**:823-846.

71. Ravetch JV, Bolland S. IgG Fc receptors. Annu Rev Immunol 2001;**19**:275-290.
72. Rus H, Cudrici C, Niculescu F. The role of the complement system in innate immunity. Immunol Res 2005;**33**:103-112.
73. Janeway CAJ. Immunobiology. 5th ed. Garland Publishing; 2001.
74. Mian IS, Bradwell AR, Olson AJ. Structure, function and properties of antibody binding sites. J Mol Biol 1991;**217**:133-151.
75. Fanning LJ, Connor AM, Wu GE. Development of the immunoglobulin repertoire. Clin Immunol Immunopathol 1996;**79**:1-14.
76. Nemazee D. Receptor editing in lymphocyte development and central tolerance. Nat Rev Immunol 2006;**6**:728-740.
77. Akdis CA, Akdis M, Trautmann A, Blaser K. Immune regulation in atopic dermatitis. Curr Opin Immunol 2000;**12**:641-646.
78. Akdis M, Simon HU, Weigl L, Kreyden O, Blaser K, Akdis CA. Skin homing (cutaneous lymphocyte-associated antigen-positive) CD8+ T cells respond to superantigen and contribute to eosinophilia and IgE production in atopic dermatitis. J Immunol 1999;**163**:466-475.
79. Abernathy-Carver KJ, Sampson HA, Picker LJ, Leung DY. Milk-induced eczema is associated with the expansion of T cells expressing cutaneous lymphocyte antigen. J Clin Invest 1995;**95(2)**:913-918.
80. Klunker S, Trautmann A, Akdis M, et al. A second step of chemotaxis after transendothelial migration: keratinocytes undergoing apoptosis release IFN-gamma-inducible protein 10, monokine induced by IFN-gamma, and IFN-gamma-inducible alpha-chemoattractant for T cell chemotaxis toward epidermis in atopic dermatitis. J Immunol 2003;**171**:1078-1084.
81. Luster AD. The role of chemokines in linking innate and adaptive immunity. Curr Opin Immunol 2002;**14(1)**:129-135.
82. Gutierrez-Ramos JC, Lloyd C, Kapsenberg ML, Gonzalo JA, Coyle AJ. Non-redundant functional groups of chemokines operate in a coordinate manner during the inflammatory response in the lung. Immunol Rev 2000;**177**:31-42.
83. Akdis M, Trautmann A, Klunker S, et al. T helper (Th) 2 predominance in atopic diseases is due to preferential apoptosis of circulating memory/effector Th1 cells. FASEB J 2003;**17**:1026-1035.
84. Simon HU, Blaser K. Inhibition of programmed eosinophil death: a key pathogenic event for eosinophilia? Immunol Today 1995;**16(2)**:53-55.
85. Akdis M, Akdis CA, Weigl L, Disch R, Blaser K. Skin-homing, CLA+ memory T cells are activated in atopic dermatitis and regulate IgE by an IL-13-dominated

cytokine pattern: IgG4 counter-regulation by CLA- memory T cells. J Immunol 1997;**159**:4611-4619.

86. Whittaker L, Niu N, Temann UA, et al. Interleukin-13 mediates a fundamental pathway for airway epithelial mucus induced by CD4 T cells and interleukin-9. Am J Respir Cell Mol Biol 2002;**27(5)**:593-602.
87. Akdis M, Verhagen J, Taylor A, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med 2004;**199**:1567-1575.
88. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy. J Allergy Clin Immunol 2007;**119**:780-791.
89. Mazzoni A, Young HA, Spitzer JH, Visintin A, Segal DM. Histamine regulates cytokine production in maturing dendritic cells, resulting in altered T cell polarization. J Clin Invest 2001;**108**:1865-1873.
90. Osna N, Elliott K, Khan MM. Regulation of interleukin-10 secretion by histamine in TH2 cells and splenocytes. Int Immunopharmacol 2001;**1(1)**:85-96.
91. Kunzmann S, Mantel PY, Wohlfahrt JG, Akdis M, Blaser K, Schmidt-Weber CB. Histamine enhances TGF-beta1-mediated suppression of Th2 responses. FASEB J 2003;**17**:1089-1095.
92. Jutel M, Watanabe T, Klunker S, et al. Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors. Nature 2001;**413**:420-425.
93. Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology 1970;**18**:723-737.
94. Gershon RK, Kondo K. Infectious immunological tolerance. Immunology 1971;**21**:903-914.
95. Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK. Type 1 T regulatory cells. Immunol Rev 2001;**182**:68-79.
96. Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. Immunol Rev 2001;**182**:207-214.
97. Inobe J, Slavin AJ, Komagata Y, Chen Y, Liu L, Weiner HL. IL-4 is a differentiation factor for transforming growth factor-beta secreting Th3 cells and oral administration of IL-4 enhances oral tolerance in experimental allergic encephalomyelitis. Eur J Immunol 1998;**28**:2780-2790.
98. Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. J Clin Invest 1998;**102**:98-106.
99. Barrat FJ, Cua DJ, Boonstra A, et al. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs

and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. J Exp Med 2002;**195(5)**:603-616.

100. Groux H, O'Garra A, Bigler M, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;**389**:737-742.
101. Levings MK, Sangregorio R, Galbiati F, Squadrone S, de Waal Malefyt R, Roncarolo MG. IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. J Immunol 2001;**166**:5530-5539.
102. Roncarolo MG, Levings MK, Traversari C. Differentiation of T regulatory cells by immature dendritic cells. J Exp Med 2001;**193(2)**:F5-9.
103. Walker LS, Chodos A, Eggena M, Dooms H, Abbas AK. Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. J Exp Med 2003;**198(2)**:249-258.
104. Manzotti CN, Tipping H, Perry LC, et al. Inhibition of human T cell proliferation by CTLA-4 utilizes CD80 and requires CD25+ regulatory T cells. Eur J Immunol 2002;**32(10)**:2888-2896.
105. Takahashi T, Tagami T, Yamazaki S, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med 2000;**192**:303-310.
106. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999;**11(2)**:141-151.
107. Carter L, Fouser LA, Jussif J, et al. PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2. Eur J Immunol 2002;**32**:634-643.
108. Bacchetta R, Sartirana C, Levings MK, Bordignon C, Narula S, Roncarolo MG. Growth and expansion of human T regulatory type 1 cells are independent from TCR activation but require exogenous cytokines. Eur J Immunol 2002;**32(8)**:2237-2245.
109. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;**19**:683-765.
110. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;**299(5609)**:1057-1061.
111. Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. J Exp Med 2001;**193**:1295-1302.
112. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2002;**2(6)**:389-400.

113. Taams LS, Vukmanovic-Stejic M, Smith J, et al. Antigen-specific T cell suppression by human CD4+CD25+ regulatory T cells. Eur J Immunol 2002;**32(6)**:1621-1630.
114. Tang Q, Boden EK, Henriksen KJ, Bour-Jordan H, Bi M, Bluestone JA. Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. Eur J Immunol 2004;**34(11)**:2996-3005.
115. McHugh RS, Whitters MJ, Piccirillo CA, et al. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. Immunity 2002;**16**:311-323.
116. Ji HB, Liao G, Faubion WA, et al. Cutting edge: the natural ligand for glucocorticoid-induced TNF receptor-related protein abrogates regulatory T cell suppression. J Immunol 2004;**172(10)**:5823-5827.
117. Wildin RS, Ramsdell F, Peake J, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet 2001;**27**:18-20.
118. Chatila TA, Blaeser F, Ho N, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. J Clin Invest 2000;**106**:R75-81.
119. Verhagen J, Akdis M, Traidl-Hoffmann C, et al. Absence of T-regulatory cell expression and function in atopic dermatitis skin. J Allergy Clin Immunol 2006;**117**:176-183.
120. Karagiannidis C, Akdis M, Holopainen P, et al. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. J Allergy Clin Immunol 2004;**114**:1425-1433.
121. Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 1994;**265**:1237-1240.
122. Taylor A, Akdis M, Joss A, et al. IL-10 inhibits CD28 and ICOS costimulations of T cells via src homology 2 domain-containing protein tyrosine phosphatase 1. J Allergy Clin Immunol 2007;**120(1)**:76-83.
123. Akdis CA, Joss A, Akdis M, Faith A, Blaser K. A molecular basis for T cell suppression by IL-10: CD28-associated IL-10 receptor inhibits CD28 tyrosine phosphorylation and phosphatidylinositol 3-kinase binding. FASEB J 2000;**14**:1666-1668.
124. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 1991;**174(5)**:1209-1220.

125. Ito S, Ansari P, Sakatsume M, et al. Interleukin-10 inhibits expression of both interferon alpha- and interferon gamma- induced genes by suppressing tyrosine phosphorylation of STAT1. Blood 1999;**93(5)**:1456-1463.
126. Enk AH, Saloga J, Becker D, Mohamadzadeh M, Knop J. Induction of hapten-specific tolerance by interleukin 10 in vivo. J Exp Med 1994;**179**:1397-1402.
127. Bacchetta R, Bigler M, Touraine JL, et al. High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. J Exp Med 1994;**179**:493-502.
128. Becker JC, Czerny C, Brocker EB. Maintenance of clonal anergy by endogenously produced IL-10. Int Immunol 1994;**6**:1605-1612.
129. Jeannin P, Lecoanet S, Delneste Y, Gauchat JF, Bonnefoy JY. IgE versus IgG4 production can be differentially regulated by IL-10. J Immunol 1998;**160(7)**:3555-3561.
130. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med 2001;**194**:629-644.
131. Huber S, Schramm C, Lehr HA, et al. Cutting edge: TGF-beta signaling is required for the in vivo expansion and immunosuppressive capacity of regulatory CD4+CD25+ T cells. J Immunol 2004;**173**:6526-6531.
132. Fantini MC, Becker C, Tubbe I, et al. Transforming growth factor beta induced FoxP3+ regulatory T cells suppress Th1 mediated experimental colitis. Gut 2006;**55**:671-680.
133. Rao PE, Petrone AL, Ponath PD. Differentiation and expansion of T cells with regulatory function from human peripheral lymphocytes by stimulation in the presence of TGF-{beta}. J Immunol 2005;**174**:1446-1455.
134. Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. J Exp Med 2005;**201**:1061-1067.
135. Park H, Li Z, Yang XO, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005;**6(11)**:1133-1141.
136. Aggarwal S, Gurney AL. IL-17: prototype member of an emerging cytokine family. J Leukoc Biol 2002;**71(1)**:1-8.
137. Moseley TA, Haudenschild DR, Rose L, Reddi AH. Interleukin-17 family and IL-17 receptors. Cytokine Growth Factor Rev 2003;**14(2)**:155-174.
138. Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity 2004;**21(4)**:467-476.

139. Saito H. Much atopy about the skin: genome-wide molecular analysis of atopic eczema. Int Arch Allergy Immunol 2005;**137(4)**:319-325.
140. Strachan DP. Hay fever, hygiene, and household size. BMJ 1989;**299**:1259-1260.
141. Wills-Karp M, Santeliz J, Karp CL. The germless theory of allergic disease: revisiting the hygiene hypothesis. Nat Rev Immunol 2001;**1(1)**:69-75.
142. Umetsu DT, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH. Asthma: an epidemic of dysregulated immunity. Nat Immunol 2002;**3(8)**:715-720.
143. Noverr MC, Huffnagle GB. Does the microbiota regulate immune responses outside the gut? Trends Microbiol 2004;**12(12)**:562-568.
144. Mazmanian SK, Kasper DL. The love-hate relationship between bacterial polysaccharides and the host immune system. Nat Rev Immunol 2006;**6(11)**:849-858.
145. Romagnani S. Regulation of the development of type 2 T-helper cells in allergy. Curr Opin Immunol 1994;**6(6)**:838-846.
146. Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. Science 2002;**296(5567)**:490-494.
147. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 2002;**347(12)**:911-920.
148. Dunne DW, Cooke A. A worm's eye view of the immune system: consequences for evolution of human autoimmune disease. Nat Rev Immunol 2005;**5(5)**:420-426.
149. Maizels RM, Yazdanbakhsh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat Rev Immunol 2003;**3(9)**:733-744.
150. Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. Nat Rev Immunol 2003;**3**:169-176.
151. Janeway CAJ, Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002;**20**:197-216.
152. Sato M, Sano H, Iwaki D, et al. Direct binding of Toll-like receptor 2 to zymosan, and zymosan-induced NF-kappa B activation and TNF-alpha secretion are down-regulated by lung collectin surfactant protein A. J Immunol 2003;**171**:417-425.
153. Bell JK, Botos I, Hall PR, et al. The molecular structure of the TLR3 extracellular domain. J Endotoxin Res 2006;**12**:375-378.
154. Andersen-Nissen E, Hawn TR, Smith KD, et al. Cutting edge: Tlr5-/- mice are more susceptible to Escherichia coli urinary tract infection. J Immunol 2007;**178**:4717-4720.

155. Bauer S, Kirschning CJ, Hacker H, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci U S A 2001;**98**:9237-9242.
156. Bauer S, Hangel D, Yu P. Immunobiology of toll-like receptors in allergic disease. Immunobiology 2007;**212(6)**:521-533.
157. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;**4**:499-511.
158. Toshchakov V, Jones BW, Perera PY, et al. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat Immunol 2002;**3(4)**:392-398.
159. Hoshino K, Kaisho T, Iwabe T, Takeuchi O, Akira S. Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation. Int Immunol 2002;**14(10)**:1225-1231.
160. Doyle S, Vaidya S, O'Connell R, et al. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. Immunity 2002;**17(3)**:251-263.
161. Krug A, Luker GD, Barchet W, Leib DA, Akira S, Colonna M. Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. Blood 2004;**103(4)**:1433-1437.
162. Ito T, Amakawa R, Kaisho T, et al. Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. J Exp Med 2002;**195(11)**:1507-1512.
163. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;**392(6673)**:245-252.
164. Wagner H. Toll meets bacterial CpG-DNA. Immunity 2001;**14(5)**:499-502.
165. Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. Nat Immunol 2001;**2(10)**:947-950.
166. Langenkamp A, Messi M, Lanzavecchia A, Sallusto F. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat Immunol 2000;**1(4)**:311-316.
167. Rissoan MC, Soumelis V, Kadowaki N, et al. Reciprocal control of T helper cell and dendritic cell differentiation. Science 1999;**283(5405)**:1183-1186.
168. McCartney-Francis N, Jin W, Wahl SM. Aberrant Toll receptor expression and endotoxin hypersensitivity in mice lacking a functional TGF-beta 1 signaling pathway. J Immunol 2004;**172**:3814-3821.
169. Naiki Y, Michelsen KS, Zhang W, Chen S, Doherty TM, Arditi M. Transforming growth factor-beta differentially inhibits MyD88-dependent, but not TRAM- and

TRIF-dependent, lipopolysaccharide-induced TLR4 signaling. J Biol Chem 2005;**280**:5491-5495.

170. Akira S, Takeda K. Functions of toll-like receptors: lessons from KO mice. C R Biol 2004;**327**:581-589.
171. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature 2002;**416**:603-607.
172. Lau CM, Broughton C, Tabor AS, et al. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. J Exp Med 2005;**202**:1171-1177.
173. Christensen SR, Kashgarian M, Alexopoulou L, Flavell RA, Akira S, Shlomchik MJ. Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. J Exp Med 2005;**202**:321-331.
174. Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchik MJ. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. Immunity 2006;**25**:417-428.
175. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity 2001;**14**:617-629.
176. Balazs M, Martin F, Zhou T, Kearney J. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. Immunity 2002;**17**:341-352.
177. Macpherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H, Zinkernagel RM. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science 2000;**288**:2222-2226.
178. Fearon DT, Carroll MC. Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. Annu Rev Immunol 2000;**18**:393-422.
179. Brodeur SR, Angelini F, Bacharier LB, et al. C4b-binding protein (C4BP) activates B cells through the CD40 receptor. Immunity 2003;**18**:837-848.
180. Pasare C, Medzhitov R. Control of B-cell responses by Toll-like receptors. Nature 2005;**438(7066)**:364-368.
181. Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood 2003;**101**:4500-4504.

182. Bourke E, Bosisio D, Golay J, Polentarutti N, Mantovani A. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood 2003;**102**:956-963.
183. Wirths S, Lanzavecchia A. ABCB1 transporter discriminates human resting naive B cells from cycling transitional and memory B cells. Eur J Immunol 2005;**35**:3433-3441.
184. Ruprecht CR, Lanzavecchia A. Toll-like receptor stimulation as a third signal required for activation of human naive B cells. Eur J Immunol 2006;**36**:810-816.
185. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science 2002;**298**:2199-2202.
186. Ehlers M, Fukuyama H, McGaha TL, Aderem A, Ravetch JV. TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE. J Exp Med 2006;**203**:553-561.
187. Liu N, Ohnishi N, Ni L, Akira S, Bacon KB. CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. Nat Immunol 2003;**4**:687-693.
188. Peng SL, Szabo SJ, Glimcher LH. T-bet regulates IgG class switching and pathogenic autoantibody production. Proc Natl Acad Sci U S A 2002;**99**:5545-5550.
189. Peng SL, Li J, Lin L, Gerth A. The role of T-bet in B cells. Nat Immunol 2003;**4**:1041; author reply 1041.
190. He B, Qiao X, Cerutti A. CpG DNA induces IgG class switch DNA recombination by activating human B cells through an innate pathway that requires TLR9 and cooperates with IL-10. J Immunol 2004;**173**:4479-4491.
191. Jego G, Palucka AK, Blanck JP, Chalouni C, Pascual V, Banchereau J. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. Immunity 2003;**19**:225-234.
192. Litinskiy MB, Nardelli B, Hilbert DM, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat Immunol 2002;**3**:822-829.
193. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat Immunol 2005;**6**:769-776.
194. Dubois B, Massacrier C, Vanbervliet B, et al. Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes. J Immunol 1998;**161**:2223-2231.

195. Mora JR, Iwata M, Eksteen B, et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science 2006;**314**:1157-1160.
196. Fagarasan S, Kinoshita K, Muramatsu M, Ikuta K, Honjo T. In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. Nature 2001;**413**:639-643.
197. Takhar P, Smurthwaite L, Coker HA, et al. Allergen drives class switching to IgE in the nasal mucosa in allergic rhinitis. J Immunol 2005;**174**:5024-5032.
198. Xu W, He B, Chiu A, et al. Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. Nat Immunol 2007;**8**:294-303.
199. Peng G, Guo Z, Kiniwa Y, et al. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. Science 2005;**309(5739)**:1380-1384.
200. Sutmuller RP, den Brok MH, Kramer M, et al. Toll-like receptor 2 controls expansion and function of regulatory T cells. J Clin Invest 2006;**116(2)**:485-494.
201. von Mutius E, Pearce N, Beasley R, et al. International patterns of tuberculosis and the prevalence of symptoms of asthma, rhinitis, and eczema. Thorax 2000;**55(6)**:449-453.
202. Lauener RP, Birchler T, Adamski J, et al. Expression of CD14 and Toll-like receptor 2 in farmers' and non-farmers' children. Lancet 2002;**360(9331)**:465-466.
203. Ahmad-Nejad P, Mrabet-Dahbi S, Breuer K, et al. The toll-like receptor 2 R753Q polymorphism defines a subgroup of patients with atopic dermatitis having severe phenotype. J Allergy Clin Immunol 2004;**113(3)**:565-567.
204. Eder W, Klimecki W, Yu L, et al. Toll-like receptor 2 as a major gene for asthma in children of European farmers. J Allergy Clin Immunol 2004;**113(3)**:482-488.
205. Gereda JE, Leung DY, Thatayatikom A, et al. Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. Lancet 2000;**355(9216)**:1680-1683.
206. Braun-Fahrlander C, Riedler J, Herz U, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med 2002;**347(12)**:869-877.
207. Koppelman GH, Reijmerink NE, Colin Stine O, et al. Association of a promoter polymorphism of the CD14 gene and atopy. Am J Respir Crit Care Med 2001;**163(4)**:965-969.
208. Floistrup H, Swartz J, Bergstrom A, et al. Allergic disease and sensitization in Steiner school children. J Allergy Clin Immunol 2006;**117(1)**:59-66.

209. Valenta R, Ball T, Focke M, et al. Immunotherapy of allergic disease. Adv Immunol 2004;**82**:105-153.
210. Bateman ED, Hurd SS, Barnes PJ, et al. Global strategy for asthma management and prevention: GINA executive summary. Eur Respir J 2008;**31**:143-178.
211. Holgate ST. Asthma: more than an inflammatory disease. Curr Opin Allergy Clin Immunol 2002;**2(1)**:27-29.
212. Passalacqua G, Durham SR. Allergic rhinitis and its impact on asthma update: allergen immunotherapy. J Allergy Clin Immunol 2007;**119(4)**:881-891.
213. Varney VA, Gaga M, Frew AJ, Aber VR, Kay AB, Durham SR. Usefulness of immunotherapy in patients with severe summer hay fever uncontrolled by antiallergic drugs. BMJ 1991;**302(6771)**:265-269.
214. Durham SR, Walker SM, Varga EM, et al. Long-term clinical efficacy of grass-pollen immunotherapy. N Engl J Med 1999;**341(7)**:468-475.
215. Nouri-Aria KT, Wachholz PA, Francis JN, et al. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. J Immunol 2004;**172(5)**:3252-3259.
216. Flicker S, Steinberger P, Norderhaug L, et al. Conversion of grass pollen allergen-specific human IgE into a protective IgG(1) antibody. Eur J Immunol 2002;**32(8)**:2156-2162.
217. van Neerven RJ, Wikborg T, Lund G, et al. Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation. J Immunol 1999;**163**:2944-2952.
218. Jutel M, Akdis M, Blaser K, Akdis CA. Mechanisms of allergen specific immunotherapy--T-cell tolerance and more. Allergy 2006;**61**:796-807.
219. Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Muller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. J Immunol 1995;**154(8)**:4187-4194.
220. Bellinghausen I, Metz G, Enk AH, Christmann S, Knop J, Saloga J. Insect venom immunotherapy induces interleukin-10 production and a Th2-to-Th1 shift, and changes surface marker expression in venom-allergic subjects. Eur J Immunol 1997;**27(5)**:1131-1139.
221. Akdis CA, Blaser K. IL-10-induced anergy in peripheral T cell and reactivation by microenvironmental cytokines: two key steps in specific immunotherapy. FASEB J 1999;**13**:603-609.

222. Jutel M, Akdis M, Budak F, et al. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 2003;**33**:1205-1214.
223. Akdis M, Blaser K, Akdis CA. T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. J Allergy Clin Immunol 2005;**116**:961-8; quiz 969.
224. Ling EM, Smith T, Nguyen XD, et al. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. Lancet 2004;**363**:608-615.
225. Verhagen J, Blaser K, Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy: T-regulatory cells and more. Immunol Allergy Clin North Am 2006;**26**:207-31, vi.
226. Punnonen J, de Waal Malefyt R, van Vlasselaer P, Gauchat JF, de Vries JE. IL-10 and viral IL-10 prevent IL-4-induced IgE synthesis by inhibiting the accessory cell function of monocytes. J Immunol 1993;**151(3)**:1280-1289.
227. Sonoda E, Matsumoto R, Hitoshi Y, et al. Transforming growth factor beta induces IgA production and acts additively with interleukin 5 for IgA production. J Exp Med 1989;**170**:1415-1420.
228. Wachholz PA, Durham SR. Mechanisms of immunotherapy: IgG revisited. Curr Opin Allergy Clin Immunol 2004;**4**:313-318.
229. Walker C, Virchow JCJ, Bruijnzeel PL, Blaser K. T cell subsets and their soluble products regulate eosinophilia in allergic and nonallergic asthma. J Immunol 1991;**146(6)**:1829-1835.
230. Rosenberg HF, Phipps S, Foster PS. Eosinophil trafficking in allergy and asthma. J Allergy Clin Immunol 2007;**119(6)**:1303-10; quiz 1311-2.
231. Treter S, Luqman M. Antigen-specific T cell tolerance down-regulates mast cell responses in vivo. Cell Immunol 2000;**206**:116-124.
232. Shim JY, Kim BS, Cho SH, Min KU, Hong SJ. Allergen-specific conventional immunotherapy decreases immunoglobulin E-mediated basophil histamine releasability. Clin Exp Allergy 2003;**33**:52-57.
233. Marshall JS, Leal-Berumen I, Nielsen L, Glibetic M, Jordana M. Interleukin (IL)-10 inhibits long-term IL-6 production but not preformed mediator release from rat peritoneal mast cells. J Clin Invest 1996;**97(4)**:1122-1128.
234. Schandene L, Alonso-Vega C, Willems F, et al. B7/CD28-dependent IL-5 production by human resting T cells is inhibited by IL-10. J Immunol 1994;**152**:4368-4374.

235. Ohkawara Y, Lim KG, Xing Z, et al. CD40 expression by human peripheral blood eosinophils. J Clin Invest 1996;**97(7)**:1761-1766.
236. Larche M, Wraith DC. Peptide-based therapeutic vaccines for allergic and autoimmune diseases. Nat Med 2005;**11(4 Suppl)**:S69-76.
237. Muller U, Akdis CA, Fricker M, et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. J Allergy Clin Immunol 1998;**101**:747-754.
238. Drew AC, Eusebius NP, Kenins L, et al. Hypoallergenic variants of the major latex allergen Hev b 6.01 retaining human T lymphocyte reactivity. J Immunol 2004;**173(9)**:5872-5879.
239. Smith TR, Alexander C, Kay AB, Larche M, Robinson DS. Cat allergen peptide immunotherapy reduces CD4(+) T cell responses to cat allergen but does not alter suppression by CD4(+) CD25(+) T cells: a double-blind placebo-controlled study. Allergy 2004;**59(10)**:1097-1101.
240. Zuleger CL, Gao X, Burger MS, Chu Q, Payne LG, Chen D. Peptide induces CD4(+)CD25+ and IL-10+ T cells and protection in airway allergy models. Vaccine 2005;**23**:3181-3186.
241. Focke M, Linhart B, Hartl A, et al. Non-anaphylactic surface-exposed peptides of the major birch pollen allergen, Bet v 1, for preventive vaccination. Clin Exp Allergy 2004;**34(10)**:1525-1533.
242. Karamloo F, Schmid-Grendelmeier P, Kussebi F, et al. Prevention of allergy by a recombinant multi-allergen vaccine with reduced IgE binding and preserved T cell epitopes. Eur J Immunol 2005;**35(11)**:3268-3276.
243. Kussebi F, Karamloo F, Rhyner C, et al. A major allergen gene-fusion protein for potential usage in allergen-specific immunotherapy. J Allergy Clin Immunol 2005;**115**:323-329.
244. Raz E, Tighe H, Sato Y, et al. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. Proc Natl Acad Sci U S A 1996;**93(10)**:5141-5145.
245. Hsu CH, Chua KY, Tao MH, et al. Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness in vivo by genetic immunization. Nat Med 1996;**2(5)**:540-544.
246. Slater JE, Paupore E, Zhang YT, Colberg-Poley AM. The latex allergen Hev b 5 transcript is widely distributed after subcutaneous injection in BALB/c mice of its DNA vaccine. J Allergy Clin Immunol 1998;**102(3)**:469-475.

247. Golden DB, Meyers DA, Kagey-Sobotka A, Valentine MD, Lichtenstein LM. Clinical relevance of the venom-specific immunoglobulin G antibody level during immunotherapy. J Allergy Clin Immunol 1982;**69(6)**:489-493.
248. Muller U, Helbling A, Bischof M. Predictive value of venom-specific IgE, IgG and IgG subclass antibodies in patients on immunotherapy with honey bee venom. Allergy 1989;**44**:412-418.
249. Wilson DR, Lima MT, Durham SR. Sublingual immunotherapy for allergic rhinitis: systematic review and meta-analysis. Allergy 2005;**60(1)**:4-12.
250. Dy M, Lebel B, Kamoun P, Hamburger J. Histamine production during the anti-allograft response. Demonstration of a new lymphokine enhancing histamine synthesis. J Exp Med 1981;**153(2)**:293-309.
251. Kubo Y, Nakano K. Regulation of histamine synthesis in mouse CD4+ and CD8+ T lymphocytes. Inflamm Res 1999;**48**:149-153.
252. Radvany Z, Darvas Z, Kerekes K, et al. H1 histamine receptor antagonist inhibits constitutive growth of Jurkat T cells and antigen-specific proliferation of ovalbumin-specific murine T cells. Semin Cancer Biol 2000;**10(1)**:41-45.
253. Saxena SP, Brandes LJ, Becker AB, Simons KJ, LaBella FS, Gerrard JM. Histamine is an intracellular messenger mediating platelet aggregation. Science 1989;**243(4898)**:1596-1599.
254. Jutel M, Watanabe T, Akdis M, Blaser K, Akdis CA. Immune regulation by histamine. Curr Opin Immunol 2002;**14**:735-740.
255. Dy M, Schneider E. Histamine-cytokine connection in immunity and hematopoiesis. Cytokine Growth Factor Rev 2004;**15**:393-410.
256. Akdis CA, Blaser K. Histamine in the immune regulation of allergic inflammation. J Allergy Clin Immunol 2003;**112**:15-22.
257. Schneider E, Rolli-Derkinderen M, Arock M, Dy M. Trends in histamine research: new functions during immune responses and hematopoiesis. Trends Immunol 2002;**23(5)**:255-263.
258. MacGlashan DJ. Histamine: A mediator of inflammation. J Allergy Clin Immunol 2003;**112(4 Suppl)**:S53-9.
259. Haas H, Panula P. The role of histamine and the tuberomamillary nucleus in the nervous system. Nat Rev Neurosci 2003;**4(2)**:121-130.
260. Higuchi M, Yanai K, Okamura N, et al. Histamine H(1) receptors in patients with Alzheimer's disease assessed by positron emission tomography. Neuroscience 2000;**99(4)**:721-729.
261. Yokoyama H. The role of central histaminergic neuron system as an anticonvulsive mechanism in developing brain. Brain Dev 2001;**23(7)**:542-547.

262. Le Coniat M, Traiffort E, Ruat M, Arrang JM, Berger R. Chromosomal localization of the human histamine H1-receptor gene. Hum Genet 1994;**94**:186-188.
263. Nakamura T, Itadani H, Hidaka Y, Ohta M, Tanaka K. Molecular cloning and characterization of a new human histamine receptor, HH4R. Biochem Biophys Res Commun 2000;**279**:615-620.
264. Jablonowski JA, Grice CA, Chai W, et al. The first potent and selective non-imidazole human histamine H4 receptor antagonists. J Med Chem 2003;**46**:3957-3960.
265. Thurmond RL, Desai PJ, Dunford PJ, et al. A potent and selective histamine H4 receptor antagonist with anti-inflammatory properties. J Pharmacol Exp Ther 2004;**309**:404-413.
266. Leurs R, Smit MJ, Timmerman H. Molecular pharmacological aspects of histamine receptors. Pharmacol Ther 1995;**66(3)**:413-463.
267. Smit MJ, Hoffmann M, Timmerman H, Leurs R. Molecular properties and signalling pathways of the histamine H1 receptor. Clin Exp Allergy 1999;**29 Suppl 3**:19-28.
268. Togias A. H1-receptors: localization and role in airway physiology and in immune functions. J Allergy Clin Immunol 2003;**112(4 Suppl)**:S60-8.
269. Bayram H, Devalia JL, Khair OA, et al. Effect of loratadine on nitrogen dioxide-induced changes in electrical resistance and release of inflammatory mediators from cultured human bronchial epithelial cells. J Allergy Clin Immunol 1999;**104(1)**:93-99.
270. Jeannin P, Delneste Y, Gosset P, et al. Histamine induces interleukin-8 secretion by endothelial cells. Blood 1994;**84(7)**:2229-2233.
271. Meretey K, Falus A, Taga T, Kishimoto T. Histamine influences the expression of the interleukin-6 receptor on human lymphoid, monocytoid and hepatoma cell lines. Agents Actions 1991;**33**:189-191.
272. Vannier E, Dinarello CA. Histamine enhances interleukin (IL)-1-induced IL-1 gene expression and protein synthesis via H2 receptors in peripheral blood mononuclear cells. Comparison with IL-1 receptor antagonist. J Clin Invest 1993;**92(1)**:281-287.
273. Kubes P, Kanwar S. Histamine induces leukocyte rolling in post-capillary venules. A P-selectin-mediated event. J Immunol 1994;**152(7)**:3570-3577.
274. Lo WW, Fan TP. Histamine stimulates inositol phosphate accumulation via the H1-receptor in cultured human endothelial cells. Biochem Biophys Res Commun 1987;**148(1)**:47-53.

275. Yamaki K, Thorlacius H, Xie X, Lindbom L, Hedqvist P, Raud J. Characteristics of histamine-induced leukocyte rolling in the undisturbed microcirculation of the rat mesentery. Br J Pharmacol 1998;**123(3)**:390-399.
276. Schaefer U, Schmitz V, Schneider A, Neugebauer E. Histamine induced homologous and heterologous regulation of histamine receptor subtype mRNA expression in cultured endothelial cells. Shock 1999;**12**:309-315.
277. Fadel R, Herpin-Richard N, Rihoux JP, Henocq E. Inhibitory effect of cetirizine 2HCl on eosinophil migration in vivo. Clin Allergy 1987;**17(4)**:373-379.
278. Clark RA, Sandler JA, Gallin JI, Kaplan AP. Histamine modulation of eosinophil migration. J Immunol 1977;**118**:137-145.
279. Seligmann BE, Fletcher MP, Gallin JI. Histamine modulation of human neutrophil oxidative metabolism, locomotion, degranulation, and membrane potential changes. J Immunol 1983;**130**:1902-1909.
280. Dimitriadou V, Rouleau A, Dam Trung Tuong M, et al. Functional relationship between mast cells and C-sensitive nerve fibres evidenced by histamine H3-receptor modulation in rat lung and spleen. Clin Sci (Lond) 1994;**87(2)**:151-163.
281. Zhu Y, Michalovich D, Wu H, et al. Cloning, expression, and pharmacological characterization of a novel human histamine receptor. Mol Pharmacol 2001;**59**:434-441.
282. Hofstra CL, Desai PJ, Thurmond RL, Fung-Leung WP. Histamine H4 receptor mediates chemotaxis and calcium mobilization of mast cells. J Pharmacol Exp Ther 2003;**305**:1212-1221.
283. Idzko M, la Sala A, Ferrari D, et al. Expression and function of histamine receptors in human monocyte-derived dendritic cells. J Allergy Clin Immunol 2002;**109(5)**:839-846.
284. Gutzmer R, Langer K, Lisewski M, et al. Expression and function of histamine receptors 1 and 2 on human monocyte-derived dendritic cells. J Allergy Clin Immunol 2002;**109(3)**:524-531.
285. Caron G, Delneste Y, Roelandts E, et al. Histamine polarizes human dendritic cells into Th2 cell-promoting effector dendritic cells. J Immunol 2001;**167**:3682-3686.
286. Caron G, Delneste Y, Roelandts E, et al. Histamine induces CD86 expression and chemokine production by human immature dendritic cells. J Immunol 2001;**166**:6000-6006.
287. van der Pouw Kraan TC, Snijders A, Boeije LC, et al. Histamine inhibits the production of interleukin-12 through interaction with H2 receptors. J Clin Invest 1998;**102(10)**:1866-1873.

288. Elenkov IJ, Webster E, Papanicolaou DA, Fleisher TA, Chrousos GP, Wilder RL. Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. J Immunol 1998;**161**:2586-2593.
289. Takahashi HK, Iwagaki H, Mori S, Yoshino T, Tanaka N, Nishibori M. Histamine inhibits lipopolysaccharide-induced interleukin (IL)-18 production in human monocytes. Clin Immunol 2004;**112(1)**:30-34.
290. Morichika T, Takahashi HK, Iwagaki H, et al. Histamine inhibits lipopolysaccharide-induced tumor necrosis factor-alpha production in an intercellular adhesion molecule-1- and B7.1-dependent manner. J Pharmacol Exp Ther 2003;**304(2)**:624-633.
291. Takahashi HK, Morichika T, Iwagaki H, et al. Histamine downregulates CD14 expression via H2 receptors on human monocytes. Clin Immunol 2003;**108(3)**:274-281.
292. Ohtani T, Aiba S, Mizuashi M, Mollah ZU, Nakagawa S, Tagami H. H1 and H2 histamine receptors are absent on Langerhans cells and present on dermal dendritic cells. J Invest Dermatol 2003;**121(5)**:1073-1079.
293. Borkowski TA, Letterio JJ, Mackall CL, et al. A role for TGFbeta1 in langerhans cell biology. Further characterization of the epidermal Langerhans cell defect in TGFbeta1 null mice. J Clin Invest 1997;**100(3)**:575-581.
294. Soga F, Katoh N, Kishimoto S. Histamine prevents apoptosis in human monocytes. Clin Exp Allergy 2007;**37(3)**:323-330.
295. Bakker RA, Schoonus SB, Smit MJ, Timmerman H, Leurs R. Histamine H(1)-receptor activation of nuclear factor-kappa B: roles for G beta gamma- and G alpha(q/11)-subunits in constitutive and agonist-mediated signaling. Mol Pharmacol 2001;**60(5)**:1133-1142.
296. Gelfand EW, Cui ZH, Takeda K, Kanehiro A, Joetham A. Fexofenadine modulates T-cell function, preventing allergen-induced airway inflammation and hyperresponsiveness. J Allergy Clin Immunol 2002;**110(1)**:85-95.
297. Gifford RR, Schmidtke JR. Cimetidine-induced augmentation of human lymphocyte blastogenesis: comparison with levamisole in mitogen stimulation. Surg Forum 1979;**30**:113-115.
298. Lefranc F, Yeaton P, Brotchi J, Kiss R. Cimetidine, an unexpected anti-tumor agent, and its potential for the treatment of glioblastoma (review). Int J Oncol 2006;**28(5)**:1021-1030.
299. Finkelman FD, Vercelli D. Advances in asthma, allergy mechanisms, and genetics in 2006. J Allergy Clin Immunol 2007

300. Romagnani S. Immunologic influences on allergy and the TH1/TH2 balance. J Allergy Clin Immunol 2004;**113**:395-400.
301. Aalberse RC, Van Milligen F, Tan KY, Stapel SO. Allergen-specific IgG4 in atopic disease. Allergy 1993;**48**:559-569.
302. Rossi RE, Monasterolo G, Coco G, Silvestro L, Operti D. Evaluation of serum IgG4 antibodies specific to grass pollen allergen components in the follow up of allergic patients undergoing subcutaneous and sublingual immunotherapy. Vaccine 2007;**25**:957-964.
303. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;**4**:330-336.
304. Chatila TA. Role of regulatory T cells in human diseases. J Allergy Clin Immunol 2005;**116**:949-59; quiz 960.
305. Cameron L, Gounni AS, Frenkiel S, Lavigne F, Vercelli D, Hamid Q. S epsilon S mu and S epsilon S gamma switch circles in human nasal mucosa following ex vivo allergen challenge: evidence for direct as well as sequential class switch recombination. J Immunol 2003;**171**:3816-3822.
306. Takhar P, Corrigan CJ, Smurthwaite L, et al. Class switch recombination to IgE in the bronchial mucosa of atopic and nonatopic patients with asthma. J Allergy Clin Immunol 2007;**119**:213-218.
307. Kaisho T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immunol 2006;**117**:979-87; quiz 988.
308. Lane P, Brocker T, Hubele S, Padovan E, Lanzavecchia A, McConnell F. Soluble CD40 ligand can replace the normal T cell-derived CD40 ligand signal to B cells in T cell-dependent activation. J Exp Med 1993;**177**:1209-1213.
309. Coyle AJ, Wagner K, Bertrand C, Tsuyuki S, Bews J, Heusser C. Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: inhibition by a non-anaphylactogenic anti-IgE antibody. J Exp Med 1996;**183**:1303-1310.
310. Curotto de Lafaille MA, Lafaille JJ. CD4(+) regulatory T cells in autoimmunity and allergy. Curr Opin Immunol 2002;**14**:771-778.
311. Seo SJ, Fields ML, Buckler JL, et al. The impact of T helper and T regulatory cells on the regulation of anti-double-stranded DNA B cells. Immunity 2002;**16**:535-546.
312. Akdis M. Healthy immune response to allergens: T regulatory cells and more. Curr Opin Immunol 2006;**18**:738-744.

313. Karlsson MR, Rugtveit J, Brandtzaeg P. Allergen-responsive CD4+CD25+ regulatory T cells in children who have outgrown cow's milk allergy. J Exp Med 2004;**199**:1679-1688.
314. Platts-Mills T, Vaughan J, Squillace S, Woodfolk J, Sporik R. Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. Lancet 2001;**357**:752-756.
315. Akdis CA, Akdis M, Blesken T, et al. Epitope-specific T cell tolerance to phospholipase A2 in bee venom immunotherapy and recovery by IL-2 and IL-15 in vitro. J Clin Invest 1996;**98**:1676-1683.
316. Ejrnaes AM, Svenson M, Lund G, Larsen JN, Jacobi H. Inhibition of rBet v 1-induced basophil histamine release with specific immunotherapy -induced serum immunoglobulin G: no evidence that FcgammaRIIB signalling is important. Clin Exp Allergy 2006;**36**:273-282.
317. Jutel M, Jaeger L, Suck R, Meyer H, Fiebig H, Cromwell O. Allergen-specific immunotherapy with recombinant grass pollen allergens. J Allergy Clin Immunol 2005;**116**:608-613.
318. Radbruch A, Muehlinghaus G, Luger EO, et al. Competence and competition: the challenge of becoming a long-lived plasma cell. Nat Rev Immunol 2006;**6**:741-750.
319. Daniel C, Repa A, Wild C, et al. Modulation of allergic immune responses by mucosal application of recombinant lactic acid bacteria producing the major birch pollen allergen Bet v 1. Allergy 2006;**61(7)**:812-819.
320. He B, Xu W, Santini PA, et al. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. Immunity 2007;**26(6)**:812-826.
321. Tezuka H, Abe Y, Iwata M, et al. Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. Nature 2007;**448(7156)**:929-933.
322. Liu Z, Geboes K, Hellings P, et al. B7 interactions with CD28 and CTLA-4 control tolerance or induction of mucosal inflammation in chronic experimental colitis. J Immunol 2001;**167(3)**:1830-1838.
323. Ono M, Shimizu J, Miyachi Y, Sakaguchi S. Control of autoimmune myocarditis and multiorgan inflammation by glucocorticoid-induced TNF receptor family-related protein(high), Foxp3-expressing CD25+ and CD25- regulatory T cells. J Immunol 2006;**176(8)**:4748-4756.
324. Kitazawa Y, Fujino M, Wang Q, et al. Involvement of the programmed death-1/programmed death-1 ligand pathway in CD4+CD25+ regulatory T-cell activity to suppress alloimmune responses. Transplantation 2007;**83(6)**:774-782.

325. Takeda I, Ine S, Killeen N, et al. Distinct roles for the OX40-OX40 ligand interaction in regulatory and nonregulatory T cells. J Immunol 2004;**172(6)**:3580-3589.
326. Bopp T, Becker C, Klein M, et al. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. J Exp Med 2007
327. Schlaepfer E, Audige A, von Beust B, et al. CpG oligodeoxynucleotides block human immunodeficiency virus type 1 replication in human lymphoid tissue infected ex vivo. J Virol 2004;**78(22)**:12344-12354.
328. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;**136(7)**:2348-2357.
329. Sakaguchi S, Powrie F. Emerging challenges in regulatory T cell function and biology. Science 2007;**317(5838)**:627-629.
330. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. Nat Rev Immunol 2007;**7(8)**:585-598.
331. Hill JA, Benoist C, Mathis D. Treg cells: guardians for life. Nat Immunol 2007;**8(2)**:124-125.
332. Faria AM, Weiner HL. Oral tolerance. Immunol Rev 2005;**206**:232-259.
333. Shevach EM, DiPaolo RA, Andersson J, Zhao DM, Stephens GL, Thornton AM. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. Immunol Rev 2006;**212**:60-73.
334. Qin S, Cobbold SP, Pope H, et al. "Infectious" transplantation tolerance. Science 1993;**259(5097)**:974-977.
335. Cong Y, Weaver CT, Lazenby A, Elson CO. Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora. J Immunol 2002;**169(11)**:6112-6119.
336. Akdis CA, Blaser K, Akdis M. Genes of tolerance. Allergy 2004;**59**:897-913.
337. Peek EJ, Richards DF, Faith A, et al. Interleukin-10-secreting "regulatory" T cells induced by glucocorticoids and beta2-agonists. Am J Respir Cell Mol Biol 2005;**33(1)**:105-111.
338. Larche M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. Nat Rev Immunol 2006;**6**:761-771.
339. Bilo BM, Rueff F, Mosbech H, Bonifazi F, Oude-Elberink JN. Diagnosis of Hymenoptera venom allergy. Allergy 2005;**60(11)**:1339-1349.
340. Tarzi M, Klunker S, Texier C, et al. Induction of interleukin-10 and suppressor of cytokine signalling-3 gene expression following peptide immunotherapy. Clin Exp Allergy 2006;**36**:465-474.

341. Van Parijs L, Abbas AK. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. Science 1998;**280(5361)**:243-248.
342. Vukmanovic-Stejic M, Zhang Y, Cook JE, et al. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. J Clin Invest 2006;**116(9)**:2423-2433.
343. Sundrud MS, Grill SM, Ni D, et al. Genetic reprogramming of primary human T cells reveals functional plasticity in Th cell differentiation. J Immunol 2003;**171**:3542-3549.
344. Messi M, Giacchetto I, Nagata K, Lanzavecchia A, Natoli G, Sallusto F. Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. Nat Immunol 2003;**4(1)**:78-86.
345. Texier C, Pouvelle S, Busson M, et al. HLA-DR restricted peptide candidates for bee venom immunotherapy. J Immunol 2000;**164**:3177-3184.
346. Maurer M, Seidel-Guyenot W, Metz M, Knop J, Steinbrink K. Critical role of IL-10 in the induction of low zone tolerance to contact allergens. J Clin Invest 2003;**112(3)**:432-439.
347. Wakkach A, Fournier N, Brun V, Breittmayer JP, Cottrez F, Groux H. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. Immunity 2003;**18(5)**:605-617.
348. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med 2000;**192**:295-302.
349. Brosterhus H, Brings S, Leyendeckers H, et al. Enrichment and detection of live antigen-specific CD4(+) and CD8(+) T cells based on cytokine secretion. Eur J Immunol 1999;**29(12)**:4053-4059.
350. Liu Y, Wei SH, Ho AS, de Waal Malefyt R, Moore KW. Expression cloning and characterization of a human IL-10 receptor. J Immunol 1994;**152(4)**:1821-1829.
351. Kunzmann S, Wohlfahrt JG, Itoh S, et al. SARA and Hgs attenuate susceptibility to TGF-beta1-mediated T cell suppression. FASEB J 2003;**17**:194-202.
352. Mothes N, Heinzkill M, Drachenberg KJ, et al. Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. Clin Exp Allergy 2003;**33(9)**:1198-1208.
353. Kepley CL, Cambier JC, Morel PA, et al. Negative regulation of FcepsilonRI signaling by FcgammaRII costimulation in human blood basophils. J Allergy Clin Immunol 2000;**106(2)**:337-348.

354. Mudde GC, Van Reijsen FC, Boland GJ, de Gast GC, Bruijnzeel PL, Bruijnzeel-Koomen CA. Allergen presentation by epidermal Langerhans' cells from patients with atopic dermatitis is mediated by IgE. Immunology 1990;**69**:335-341.
355. Mudde GC, Bheekha R, Bruijnzeel-Koomen CA. IgE-mediated antigen presentation. Allergy 1995;**50(3)**:193-199.
356. Carballido JM, Faith A, Carballido-Perrig N, Blaser K. The intensity of T cell receptor engagement determines the cytokine pattern of human allergen-specific T helper cells. Eur J Immunol 1997;**27(2)**:515-521.
357. Casale TB, Busse WW, Kline JN, et al. Omalizumab pretreatment decreases acute reactions after rush immunotherapy for ragweed-induced seasonal allergic rhinitis. J Allergy Clin Immunol 2006;**117(1)**:134-140.
358. Stock P, Rolinck-Werninghaus C, Wahn U, Hamelmann E. The role of anti-IgE therapy in combination with allergen specific immunotherapy for seasonal allergic rhinitis. BioDrugs 2007;**21**:403-410.
359. Wachholz PA, Durham SR. Induction of 'blocking' IgG antibodies during immunotherapy. Clin Exp Allergy 2003;**33(9)**:1171-1174.
360. Lessof MH, Sobotka AK, Lichtenstein LM. Protection against anaphylaxis in hymenoptera-sensitive patients by passive immunization. Monogr Allergy 1977;**12**:253-256.
361. Lessof MH, Sobotka AK, Lichtenstein LM. Effects of passive antibody in bee venom anaphylaxis. Johns Hopkins Med J 1978;**142(1)**:1-7.
362. Bohle B. CpG motifs as possible adjuvants for the treatment of allergic diseases. Int Arch Allergy Immunol 2002;**129(3)**:198-203.
363. Akdis CA. Allergy and hypersensitivity: mechanisms of allergic disease. Curr Opin Immunol 2006;**18**:718-726.